REMARKS

Claims 1-5, 8, 10, and 37-51 are pending and under examination in the subject application.

Rejections under 35 U.S.C. §112, Second Paragraph

Claims 2-3, 38-39 and 50-51 are rejected under 35 U.S.C. §112, second paragraph, because the claims recite "BRL-47672." The Examiner indicated that the claim scope is uncertain because a trade name cannot be used to properly identify any particular material.

Applicants respectfully traverse this rejection.

The term BRL-47672 designates a specific compound known in the art. The chemical structure of BRL-47672 is illustrated for example in Figure 1 in each of Sillence, M.N. et al. (Am. J. Physiol. 268: E159-E167, 1995) and Jones, S.W. et al. (J. Pharmacol. Exp. Ther. 311(3):1225-1231, 2004), a copy of each is attached hereto.

Accordingly, reconsideration and withdrawal of this ground of rejection are respectfully requested.

Rejections under 35 U.S.C. §102

Claims 1, 5, 8, 37, 41-44, and 47-49 are rejected under 35 U.S.C. §102(a) as anticipated by Murphy et al. (Arch. Phys. Med. Rehabil. 80(10): 1264-67, 1999).

Applicants respectfully traverse this rejection.

Applicants note that "[a] single prior art reference anticipates a patent claim if it expressly or inherently describes each and every limitation set forth in the patent claim. *Verdegaal Bros., Inc. v. Union Oil Co.*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). Inherent anticipation requires that the missing descriptive material is

"necessarily present," not merely probably or possibly present, in the prior art. *In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999) (citing *Continental Can Co. USA, Inc. v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 USPQ2d 1746, 1749 (Fed. Cir. 1991))." *Trintec Industries, Inc. v. Top-U.S.A. Corp.*, 295 F.3d 1292, 1295, 63 USPQ2d 1597, 1599 (Fed. Cir. 1992).

Murphy et al. teach that administration of salbutamol to paralyzed subjects (one paraplegic and two tetraplegic) increased leg circumference and vastus lateralis cross-sectional area in subjects undergoing cycling artificially induced by functional electrical stimulation. Murphy et al. do not teach that salbutamol treats spinal cord tissue as opposed to muscle tissue.

The present application teaches that β_2 adrenergic agonists are effective to treat spinal cord tissue. For example, following spinal cord contusion injury, clenbuterol significantly increased the relative amount of spared spinal cord tissue (page 14, second paragraph). The extent of locomotor recovery in turn was found to be directly related to the amount of tissue spared at the injury site (page 14, lines 20-21; page 15, lines 4-6). Furthermore, in motor neuron degeneration mice, clenbuterol reduced the frequency of abnormal motoneurons, indicating a neurotrophic effect of β_2-agonist treatment (page 18, lines 24-25). Claims 47-49 explicitly require that the β_2 adrenergic agonist reduce injury-induced loss of spinal cord tissue.

There can be a critical postinjury period for treating the injured spinal cord. For example, Young (1993) reported that spinal cord injury can be treated within an eight hour postinjury therapeutic time-window using the synthetic glucocorticoid methylprednisolone (J. Emergency Med. 11:13-22, 1993, copy enclosed; see especially Abstract and section on "Treatment Initiation Time on page 16). In contrast, increasing muscle mass is not known to be restricted by a therapeutic time-window. Accordingly, it

is not "necessarily present" (as opposed to probably or possibly present) in the teachings of Murphy et al. that, just because patients had both an injured spinal cord and muscle atrophy, salbutamol treats spinal cord tissue and increases locomotor function in addition to treating muscle atrophy.

Applicants further note that independent claim 37, and accordingly dependent claims 8, 41, 44, 48, require that the subject have a "spinal cord contusion to the lower thoracic spine." [Emphasis added.] In contrast, the subjects in Murphy et al. either had cervical spinal injury (C6-C7 and C7-C8) or injury in the upper portion of the thoracic spinal cord at level Th4 (see Table 1 in Murphy et al.). Thoracic segment Th4 is in the upper third of the human thoracic spinal cord, which contains 12 segments. None of the subjects in Murphy et al. had a spinal cord contusion to the lower thoracic spine.

Accordingly, in view of the above remarks, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

Rejections under 35 U.S.C. §103(a)

A. Claim 10 is rejected under 35 U.S.C. §103(a) as not patentable over Murphy et al. (Arch. Phys. Med. Rehabil. 80(10): 1264-67, 1999), as applied to claims above.

Applicants respectfully traverse this rejection.

Applicants maintain that claim 37 and 41 are patentable over Murphy et al. for reasons discussed hereinabove and that claim 10, which depends from and further limits claims 37 and 41, is likewise therefore patentable over Murphy et al.

Accordingly, in view of the above remarks, applicants respectfully request that the Examiner reconsider and withdraw this ground of rejection.

B. Claims 1-5, 8, 10, and 37-51 are rejected under 35 U.S.C. §103(a) as not patentable over Stone et al. (U.S. Patent No. 5,281,607).

Applicants respectfully traverse this rejection.

Stone et al. teach the treatment of neurodegenerative diseases and/or central nervous system trauma using substances that increase expression of nerve growth factor (NGF) in the central nervous system, where the substances are a β-adrenergic agonist, an α1-adrenergic agonist, and/or an α2-adrenergic antagonist, where an α2-adrenergic antagonist is preferred (see Field of the Invention and Summary of the Invention). Although Stone et al. do list some individual β-adrenergic agonists, Stone et al. do not specifically teach or suggest the use of β_2 adrenergic agonists as opposed to β_1 adrenergic agonists.

The only working example provided by Stone et al. is a demonstration that the α2 antagonist yohimbine increases NGF mRNA in the hippocampus of the brains of rats. However, a subsequent 1994 publication by Stone and colleagues reported that, even though yohimbine increased NGF mRNA levels three fold, NGF protein levels were unaltered *in vivo* (Stone et al., Neurosci. Lett. 167: 11-13, 1994; copy enclosed). In view of this later report by Stone et al. that the treatment proposed in U.S. Patent No. 5,281,607 fails to alter NGF protein level in the brain, applicants submit that the skilled artisan would have reasonable doubts regarding the therapeutic effectiveness of the treatment taught in the Stone et al. patent.

Moreover, the progression of injury is not necessarily the same following injury to the brain as compared to injury to the spinal cord. For example, the acute inflammatory response to traumatic injury is significantly greater in the spinal cord than in the cerebral cortex of the brain (Schnell, L. et al. Eur. J. Neurosci. 11: 3648-58, 1999, copy enclosed). These authors also reported that the breakdown of the blood-brain barrier was

substantially larger in the spinal cord and that vascular damage persisted for a longer period following injury to the spinal cord than to the brain. Consequently, there is reason to believe that the brain and spinal cord may require different therapeutic approaches following injury.

Stone et al. do not provide the skilled artisan with a reasonable expectation of success that treating a subject with a β_2 adrenergic agonist following spinal cord contusion injury will increase locomotor function and neuromuscular strength as required in the claimed invention.

In view of the remarks made hereinabove, applicants submit that the claimed invention is patentable over Stone et al. Accordingly, reconsideration and withdrawal of this ground of rejection are respectfully requested.

CONCLUSIONS

In light of the above discussion, applicants respectfully request that the Examiner reconsider and withdraw the rejections in the August 4, 2004 Office Action and earnestly solicit passage of the pending claims to allowance. Should there be any minor matters preventing allowance of the subject application, the Examiner is requested to telephone the undersigned attorney.

A check is enclosed for $55.00 to cover the fee for a one month extension of time for a small entity. No other fee is deemed necessary in connection with the filing of this reply. However, if any unanticipated payment is required to maintain the pendency of this application, authorization is given to withdraw the amount of any such fee from Deposit Account No. 01-1785. Overpayments may also be credited to Deposit Account No. 01-1785.

Respectfully submitted,

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Dated: New York, New York
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By: _____
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Effects of BRL-47672 on growth, β_2-adrenoceptors, and adenylyl cyclase activation in female rats

M. N. SILLENCE, M. L. MATTHEWS, N. G. MOORE, AND M. M. REICH
Tropical Beef Centre: Commonwealth Scientific and Industrial Research Organization, Division of Tropical Animal Production, Central Queensland University and Queensland Department of Primary Industries, Rockhampton, Queensland 4702, Australia

Sillence, M. N., M. L. Matthews, N. G. Moore, and M. M. Reich. Effects of BRL-47672 on growth, β_2-adrenoceptors, and adenylyl cyclase activation in female rats. *Am. J. Physiol.* 268 (*Endocrinol. Metab.* 31): E159–E167, 1995.— The morpholine compound BRL-47672 has a chemical structure similar to that of clenbuterol and causes similar anabolic effects in rats but has no actions on β_2-adrenoceptors in vitro. It has been argued therefore that β_2-adrenoceptors do not mediate the anabolic effects of this family of compounds. In the present study BRL-47672 was shown to bind to rat β_2-adrenoceptors with low affinity (dissociation constant 16 μM) relative to clenbuterol (48 nM) and to be a very weak activator of adenylyl cyclase activity in rat skeletal muscle membranes in vitro. In contrast, acute administration of the drug to anesthetized rats in vivo caused an increase in muscle adenosine 3',5'-cyclic monophosphate output, and chronic treatment of conscious rats for >6 days caused a significant increase in weight gain (69%) accounted for by increased muscle growth. The anabolic effects of BRL-47672 were not counteracted by daily injections of the drug ICI-118551 (2 mg/day) but were prevented when the same β_2-antagonist was administered in the diet (200 mg/kg feed, equivalent to 4.3 mg/day). The β_1-adrenoceptor selective antagonist CGP-20712A fed in the diet (200 mg/kg feed) failed to attenuate the response to BRL-47672. These results support the conclusion that BRL-47672 has little direct action on β_2-adrenoceptors but suggest that the compound is metabolized rapidly in vivo to a potent β_2-agonist. Thus the stimulation of muscle growth by BRL-47672 is via β_2-adrenoceptors, with no contribution to this response from β_1- or β_3-adrenoceptor activation.

skeletal muscle; anabolic drugs; clenbuterol

IN RECENT YEARS there has been a growing interest in the anabolic properties of clenbuterol and other β_2-adrenoceptor agonists (β_2-agonists) because of their potential for increasing the efficiency of livestock production (17) and their ability to reduce skeletal muscle atrophy associated with denervation (29) and other clinically important wasting disorders (4, 24). To exploit the beneficial properties of such compounds fully, it would be useful to understand their mechanism of action.

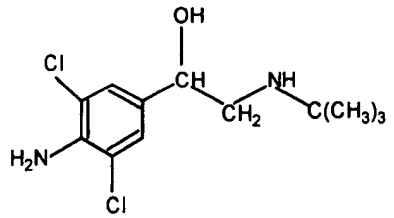
It has been argued that the anabolic effects of β_2-agonists are independent of their actions on β_2-adrenoceptors. Two reports showed that the effects of clenbuterol on muscle protein accretion were resistant to blockade by the nonselective β-adrenoceptor antagonist propranolol (12, 23), and this finding coincided with reports of a propranolol-resistant "atypical" β-adrenoceptor in rat adipose tissue (1) and soleus muscle (3, 15, 27). More debate followed the observation that salbutamol was ineffective at increasing growth rate when fed to rats (23), despite the fact that this drug is in widespread clinical use for its β_2-agonist properties. Finally, a series of morpholine compounds was synthesized, including BRL-47672, which have chemical structures and anabolic properties similar to clenbuterol (Fig. 1) but which showed no β_2-agonist activity in vitro in any test system examined (2). The morpholine compounds are of additional interest because they are claimed to be less potent stimulators of heart rate than clenbuterol (2).

Most of the arguments described above, which are against the involvement of β_2-adrenoceptors in stimulating muscle growth, have now been discredited. First, it was shown that propranolol is effective at blocking the anabolic response to clenbuterol, provided that the antagonist is given by intraperitoneal injection or orally in sufficient doses (11). More importantly, it was also demonstrated (26) and confirmed (5) that the anabolic actions of clenbuterol are prevented by concurrent administration of the β_2-adrenoceptor-selective antagonist ICI-118551. It was also shown in the former study (26) that treatment with ICI-118551 alone caused muscle atrophy, indicating that β_2-adrenoceptors have a physiological role in controlling skeletal muscle growth. Next, it was shown that salbutamol does elicit a marked anabolic effect in rats when the drug is given by continuous osmotic minipump infusion, rather than by oral administration (5), probably because salbutamol has a short plasma half-life or is poorly absorbed from the gut. Last, the atypical receptor found in adipose tissue was shown to be a new subtype of adrenoceptor, the β_3-adrenoceptor, and has been cloned from a number of species (6, 8, 19). However, most workers have failed to show the presence of mRNA for β_3-adrenoceptors in skeletal muscle (8, 10, 19).

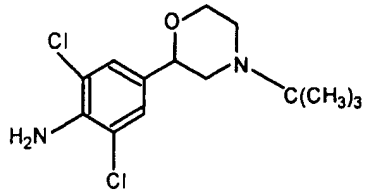
In light of these findings, the present study was designed to test the hypothesis that the anabolic effects of the morpholine compound BRL-47672 are also dependent on the activation of β_2-adrenoceptors, either directly or indirectly through metabolism of the drug in vivo to a β_2-agonist. The design of this work was based on the use of subtype-selective β-adrenoceptor antagonists, the administration of these compounds by various routes, and the awareness that skeletal muscle may contain up to three β-adrenoceptor subtypes.

MATERIALS AND METHODS

First we examined the properties of the compound BRL-47672 in vitro. In *experiment 1* the affinity of BRL-47672 for binding to β_2-adrenoceptors was determined using rat skeletal muscle membranes. In *experiment 2* the efficacy of BRL-47672 for stimulating adenylyl cyclase activation was also assessed using cell membranes from rat gastrocnemius muscle. Next we



Clenbuterol



BRL 47672

Fig. 1. Chemical structures of clenbuterol and BRL-47672.

performed experiments in vivo to determine the effects of any metabolites of BRL-47672. To investigate short-term effects on adenylyl cyclase activation in *experiments 3* and *4*, the drug was injected into anesthetized female rats and adenosine 3',5'-cyclic monophosphate (cAMP) concentrations were measured in skeletal muscle biopsy samples taken from the hindlimb over a 4-h period. *Experiments 5* and *6* were performed to determine the effects of BRL-47672 on growth. In both experiments the drug was given alone or in combination with a β-adrenoceptor antagonist over 4–6 days. In *experiment 7* we examined the effects of the β_1-adrenoceptor-selective antagonist CGP-20712A on growth, when the drug was given alone. All animal experiments were performed with the approval of the Animal Experimentation Ethics Committee of the Commonwealth Scientific and Industrial Research Organization Tropical Cattle Research Centre (Rockhampton, Australia).

Skeletal muscle membrane preparation. Gastrocnemius and soleus muscles were obtained from female rats stunned by a blow to the head and then killed by cervical dislocation. For studies of receptor binding affinity and adenylyl cyclase activation (*expts 1* and *2*), muscles obtained from eight females (180–200 g body wt) were pooled and then processed. For studies of β_2-adrenoceptor density (*expt 5*) muscles obtained from the left and right hindlimbs were pooled, but samples from individual animals were processed separately. Cell membranes were prepared by homogenization followed by centrifugation as described by Sillence et al. (27). Membrane pellets for adenylyl cyclase assays were stored dry at −80°C. The pellets used for receptor binding assays were resuspended in 1 ml of ice-cold tris(hydroxymethyl) aminomethane (Tris) assay buffer (50 mM Tris 7.7, 10 mM $MgCl_2$, 0.15 M NaCl; pH 7.4 at 37°C) and stored at −80°C. All cell membranes were used within 2 wk of preparation. Membrane protein concentration was determined using the Bradford Protein Assay (Bio-Rad, Richmond, CA), with bovine serum albumin standards.

Ligand binding assays. To determine the affinity of BRL-47672, clenbuterol, and (−)-isoproterenol for binding to β_2-adrenoceptors in rat gastrocnemius muscle membranes, three competitive binding studies were performed using each drug (*expt 1*). For each study polystyrene tubes (12 × 75 mm) were prepared in triplicate containing 50 μl (−)-[^{125}I]iodocyanopindolol ([^{125}I]ICYP; final concentration 100 pM), 50 μl 5'-guanylylimidodiphosphate, 100 μl rat muscle membrane suspension (containing 20 μg protein), and 50 μl of the competing ligand dissolved in Tris assay buffer at varying concentrations. When (−)-isoproterenol was tested, ascorbic acid (2.8 mM) was included in the buffer to retard the oxidation of this ligand. The tubes were incubated for 90 min in a shaking water bath set at 37°C and 120 cycles/min. Separation of bound from free radioligand was achieved by filtering through glass-fiber filter papers (Whatman GF-B) using a Brandel cell harvester (Beckman Instruments, Brisbane, Australia). Each tube was rinsed five times with 5 ml ice-cold Tris assay buffer. Radioactivity remaining on the filters was determined using a Gammamaster gamma counter (Linbrook, Brisbane, Australia) at a counting efficiency of 68.3%. The data were analyzed using the Ligand computer program (18), with nonspecific binding allowed to float about an initial estimate obtained from tubes incubated in the presence of 1 μM (−)-propranolol. Specific binding of [^{125}I]ICYP to β_2-adrenoceptors was between 65 and 70% of total binding.

To determine the density of β_2-adrenoceptors in membranes prepared from rat gastrocnemius muscles (*expt 5*), saturation studies were performed as described previously except that tubes contained 100 μl of membrane suspension (incubation concentration 0.2 mg protein/ml), 100 μl Tris assay buffer, and 50 μl of [^{125}I]ICYP at increasing concentrations over the range of 2–280 pM. Nonspecific binding was determined using 1 μM (−)-propranolol, and again the results were analyzed using the Ligand program (18).

Adenylyl cyclase assays. To examine the effects of BRL-47672, clenbuterol, and (−)-isoproterenol on adenylyl cyclase activity in vitro (*expt 2*), frozen cell membranes were thawed, pooled, and resuspended in 1 ml of resuspension buffer [50 mM Tris 7.0, 5 mM $MgCl_2$, 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA); pH 7.0 at 25°C] using three strokes of the tissue homogenizer. After protein determination the membrane suspension was diluted in resuspension buffer to a concentration of 0.6 mg protein/ml and left at room temperature for 30 min to prevent the lag phase in cAMP production, which would otherwise occur at the beginning of the incubation. The drugs used were dissolved in a drug dilution buffer (50 mM Tris 7.7, 100 μM ascorbic acid; pH 7.4 at 37°C). A third "incubation buffer" was made immediately before the incubation (50 mM Tris 7.7, 10 mM $MgCl_2$, 2 mM EGTA, 2 mM 3-isobutyl-1-methylxanthine, 0.4 mg bacitracin/ml, 6 mg bovine serum albumin/ml, 40 nM phosphocreatine, 2 mM ATP, 0.2 mM GTP, 0.6 mg creatine phosphokinase/ml). A small volume (1 ml) of the incubation buffer was reserved for the addition of 1.7 mg NaF.

All incubations were performed in quadruplicate. Glass tubes (12 × 75 mm) were prepared containing 50 μl of incubation buffer and 25 μl of drug solution. The incubation was initiated by the addition of 25 μl of membrane suspension and was carried out for 10 min in a shaking water bath set at 30°C. To determine the basal level of adenylyl cyclase activity, one set of tubes contained 25 μl of drug-dilution buffer with no drug added. A second set of tubes was prepared to determine maximum adenylyl cyclase activity. These contained no drug but 50 μl NaF incubation buffer instead of standard incubation buffer.

At the end of the incubation period the tubes were placed into a bath of boiling water for 4 min. Next, they were transferred to an ice-cold water bath and allowed to cool. The extraction of cAMP from the samples was performed by adding 2 ml of ice-cold 65% ethanol to all tubes, mixing, and then leaving the tubes to stand for 5 min at 4°C. The tubes were then centrifuged for 15 min at 2,000 *g* and 4°C. The supernatant was transferred to a second glass tube, and a further 1 ml of cold ethanol was added to the pellet. The tubes containing pellet were vortexed and centrifuged again for 15 min at 2,000 *g*. Finally, the supernatants from both extractions were combined and evaporated in a vacuum oven overnight at 60°C.

To determine cAMP concentrations in samples of skeletal muscle obtained from anesthetized rats (*expts 3* and *4*), 10–40 mg of muscle were homogenized in glass tubes (12 × 75 mm) in the presence of 0.5 ml ice-cold 6% trichloroacetic acid (TCA). The homogenizer head was rinsed using a further 0.5 ml TCA, before the homogenate and washings were centrifuged for 20 min at 2,000 *g* and 4°C.

The supernatant was decanted into 15 × 125 mm glass tubes, and the pellet was redissolved in 0.5 ml distilled water and centrifuged a second time for 20 min at 2,000 *g* and 4°C. The supernatant water extract was added to the TCA extract and then washed three times with five volumes of ice-cold water-saturated ether. After each wash the ether layer was removed by aspiration and discarded. The remaining aqueous phase was finally transferred to 12 × 75 mm glass tubes and then dried overnight in a vacuum oven set at 60°C.

Dried samples obtained from membrane incubations or muscle extractions were assayed for cAMP concentration using a commercially available radioimmunoassay kit (Amersham, North Ryde, Australia), after being resuspended in an assay buffer supplied with the kit.

Animal procedures: adenylyl cyclase experiments. Two experiments (*expts 3* and *4*) were performed to determine the effects of BRL-47672 on cAMP production in anesthetized rats. In both experiments female Wistar rats (180–200 g body wt) were obtained from the University of Queensland, Central Animal Breeding Laboratories (Pinjarra Hills, Brisbane, Australia) and were anesthetized using an injection (ip) of 100 μg ketamine/g body wt plus 20 μg xylazine/g body wt.

In *experiment 3* we examined the time course of the cAMP response to BRL-47672, which was expected to be delayed according to the rate at which the drug is metabolized to an active β_2-adrenoceptor agonist. In this experiment we compared BRL-47672 with the direct-acting β_2-adrenoceptor agonist clenbuterol. Accordingly, six rats per treatment were assigned randomly to three groups. Once under anesthesia animals in *group 1* were given injections (ip) of 40 μg BRL-47672/rat dissolved in 0.2 ml sterile saline, those in *group 2* were given the same dose of clenbuterol (40 μg/rat), and those in *group 3* received 0.2 ml of saline alone. The drug doses chosen were based on our previous observation that a daily dose of 0.2 mg clenbuterol/kg body wt given by injection is high enough to elicit a substantial growth response in female rats but low enough to be efficiently blocked using a moderate dose of the β_2-adrenoceptor-selective antagonist ICI-118551 (26). The difference in molecular weight between clenbuterol and BRL-47672 is <10%. Biopsy samples (~30 mg) were removed from the left lateral gastrocnemius muscle of each rat immediately before drug injection. Second and third biopsy samples were removed from the same muscle 15 and 45 min after drug injection, and further samples were taken from the right lateral gastrocnemius muscle 120 and 240 min after injection. The rats were then killed by cervical dislocation.

In *experiment 4* we examined the cAMP response to BRL-47672, given alone or in combination with ICI-118551. Again the dose of antagonist used was based on our observations from an earlier growth experiment (26), whereas the timing of antagonist injection, 15 min before injection of agonist, was based on the results of a similar experiment reported by MacLennan and Edwards (11). Thus five rats per treatment were assigned randomly to four treatment groups. *Groups 1* and *2* received injections (ip) of 1 mg ICI-118551/rat, dissolved in 0.5 ml sterile saline. Concurrently, *groups 3* and *4* received 0.5-ml injections of saline alone. Fifteen minutes later biopsy samples (~30 mg) were removed from the left gastrocnemius muscle of each rat, and a second set of injections was given. *Groups 2* and *3* received 40 μg BRL-47672/rat dissolved in 0.2 ml saline, whereas *groups 1* and *4* received 0.2 ml saline alone. After a further 90 min, a biopsy sample was taken from the right gastrocnemius muscle of each rat. Because the gastrocnemius muscle contains mixed muscle fibers, with a predominance of fast-glycolytic fibers near the surface, an additional sample was taken from the right soleus muscle at the end of the experiment to examine the response to the drugs in a muscle having slow-oxidative fibers. The rats were killed by cervical dislocation. Muscle biopsy samples taken in both experiments were weighed, snap frozen in liquid N, and stored at −80°C.

Animal procedures: growth experiments. Experiments 5 and *6* were performed using female Wistar rats to determine the effects of BRL-47672 alone, or in combination with a β-adrenoceptor antagonist, on growth. *Experiment 7* was performed to assess the effects of the β_1-antagonist CGP-20712A on growth. In all three experiments animals were housed individually in wire-bottom cages in a room controlled for light (12 h on), temperature (22°C), and humidity. The rats were allowed free access to water and to a large, but measured, quantity of a powdered laboratory diet (Norco Cooperative, Brisbane, Australia). The rats were weighed and handled daily. At the end of each experiment, feed not eaten or spilled was weighed also, so that feed intake could be estimated. Feed conversion efficiency was calculated as gram feed eaten per gram weight gain.

In *experiment 5*, 24 rats (203 ± 2.4 g body wt) were assigned to four treatment groups of similar mean body weight. The rats were given either BRL-47672, ICI-118551, a combination of both drugs, or acted as controls and received neither compound. BRL-47672 was administered by mixing with the powdered diet at a concentration of 5 mg/kg feed. ICI-118551 was administered at a dose of 1 mg/rat given by subcutaneous injection twice daily at 0800 and 1600. Animals that did not receive the antagonist were given twice daily injections of saline instead. After 6 days of treatment the rats were stunned by a blow to the head and then killed by cervical dislocation. The gastrocnemius muscles were quickly removed, weighed, and then snap frozen in liquid N and stored at −80°C until used to determine skeletal muscle β_2-adrenoceptor density. Soleus muscles and hearts were also removed, weighed, and then discarded. Finally, the bodies were eviscerated, and carcass weight was recorded along with the weight of the periuterine fat pad.

In *experiment 5* BRL-47672 appeared to downregulate β_2-adrenoceptors, but the growth response to the drug was not blocked by the β_2-antagonist. Thus *experiment 6* was performed to determine whether a higher dose of β_2-antagonist would be effective or alternatively whether β_1-adrenoceptors might be involved in the response to BRL-47672. Twenty rats (191 ± 3 g body wt) were allocated to four treatment groups balanced for live weight. The first group of rats received the standard diet and acted as controls. The other three groups were given a diet of 5 mg BRL-47672/kg feed. One of the BRL-47672-treated groups was also given the β_2-adrenoceptor-selective antagonist ICI-118551, this time presented in the feed in an attempt to prolong its bioavailability. The concentration of ICI-118551 used (200 mg/kg feed) was estimated to provide approximately twice the daily dose of antagonist given in *experiment 5*. A second group of BRL-47672-treated rats received feed containing the β_1-adrenoceptor-selective antagonist CGP-20712A (200 mg/kg feed). The third group received the agonist BRL-47672 alone. Because our supplies of ICI-118551 were limited, *experiment 6* lasted only 4 days. As in *experiment 5* the animals were killed at the end of the experiment, and weights were recorded for gastrocnemius and soleus muscles, hearts, the periuterine fat pad, and carcass.

In *experiment 6* animals treated with BRL-47672 in combination with CGP-20712A showed a consistently larger growth response than those rats treated with BRL-47672 alone, even though the difference between these groups never reached statistical significance. Thus *experiment 7* was performed to determine whether the CGP-20712A alone might cause any anabolic effects. Twelve female rats (173 ± 0.5 g body wt) were assigned to two groups of similar mean body weight. The rats were given either powdered control diet or a diet containing 200 mg CGP-20712A/kg feed. The animals were weighed daily for the following 10 days, and at the end of the experiment they were killed and their bodies dissected as described for *experiments 5* and *6*.

Chemicals. (−)-Isoproterenol, (−)-propranolol hydrochloride, and all buffer salts were from Sigma Chemical (St. Louis, MO). Bovine serum albumin was from Armour (Kanakee, IL). (−)-[^{125}I]-iodocyanopindolol (2,200 Ci/mmol) was from Dupont (North Ryde, Australia). Ketamine and xylazine were from Jurox (Riverstone, Australia). Clenbuterol was synthesized in our laboratories as described by Pegg et al. (21). The following drugs were received as gifts: CGP-20712A was from Ciba Geigy (Basel, Switzerland), ICI-118551 was from ICI (Macclesfield, UK), and BRL-47672 [4-(4-tert-butylmorpholin-2-yl)-2,6-dichlorophenylamine] was from SmithKline Beecham (Epsom, UK).

Statistics. Values are presented as means ± SE. Where comparisons were made between two groups, Student's *t*-test was used. For multiple comparisons analysis of variance (ANOVA) was performed, followed by Tukey's test to achieve means separation if appropriate.

RESULTS

Affinity of BRL-47672 for binding to β_2-adrenoceptors (expt 1). The displacement of [^{125}I]ICYP from β_2-adrenoceptors was monophasic (Fig. 2), and Hill coefficients were close to unity for all three ligands, consistent with their binding to a single class of binding sites. The dissociation constant (K_D) value calculated for each drug is equivalent to the concentration of that drug predicted to occupy 50% of the binding sites in the absence of [^{125}I]ICYP or any other competitor. The K_D values obtained for clenbuterol, (−)-isoproterenol, and BRL-47672 were 48 ± 6, 960 ± 60, and 16,000 ± 5,000 nM, respectively, and are consistent with the binding of these drugs to β_2-adrenoceptors.

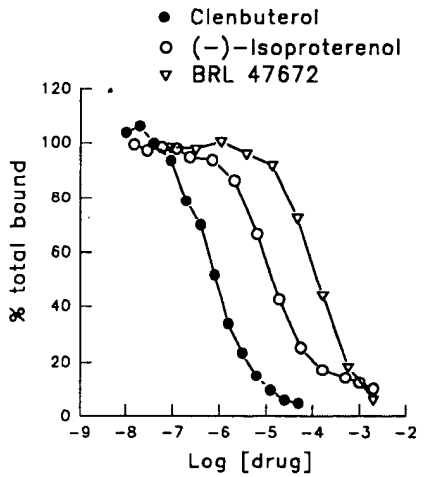


Fig. 2. Representative curves showing displacement of (−)-[^{125}I]iodocyanopindolol ([^{125}I]ICYP; 100 pM) from rat β_2-adrenoceptors by 3 competing ligands (*expt 1*). Nonspecific binding, determined using 1 μM (−)-propranolol, was 30–35% of total binding and has been subtracted from data. Each point represents mean of 3 observations.

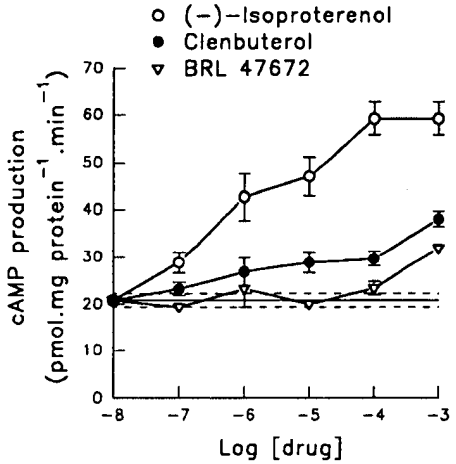


Fig. 3. Concentration-response curves of drug-induced increases in cAMP production elicited in crude cell membranes prepared from rat skeletal muscle (*expt 2*). Each point represents mean ± SE of 4 observations. SE values that are not visible are smaller than symbols. Cell membranes were incubated in presence of drugs for 10 min. Mean ± SE basal rate of cAMP production is indicated by horizontal lines.

Second-messenger studies in vitro (expt 2). Basal and maximal (NaF-stimulated) rates of cAMP production were 20.8 ± 1.4 and 114 ± 6.2 pmol·mg protein^{-1}·min^{-1}, respectively. The effects of (−)-isoproterenol, clenbuterol, and BRL-47672 are shown in Fig. 3. (−)-Isoproterenol caused a marked and dose-related stimulation of cAMP production. The maximum response to (−)-isoproterenol was seen at a concentration of 100 μM (when cAMP output plateaued), and 50% of this response (EC_{50}; judged from the graph by eye as half-way between the plateau and basal output) was produced at a concentration of ~650 nM. The latter concentration is acceptably close to the K_D for the binding of (−)-isoproterenol to β_2-adrenoceptors, determined in *experiment 1*.

The response to clenbuterol appeared to plateau at ~10 μM, before rising again at 1 mM. Based on the K_D of clenbuterol determined in *experiment 1*, 99.5% of β_2-adrenoceptors would be occupied by clenbuterol at a concentration of 10 μM, and therefore the marked increase in cAMP output seen at concentrations higher than this is not consistent with β_2-adrenoceptor activation. Instead, this latter increase probably represents a nonspecific effect of the drug. With the assumption that the plateau observed at 10 μM represents the plateau of the β_2-adrenoceptor response, the EC_{50} concentration of clenbuterol was estimated to be 350 nM, which is within one order of magnitude of the K_D for clenbuterol determined in *experiment 1*. Compared with the maximum response achieved using (−)-isoproterenol, the maximum response to clenbuterol acting via β_2-adrenoceptors was only 23%, confirming that clenbuterol is only a partial agonist at β_2-adrenoceptors.

No stimulation of cAMP output was seen in response to BRL-47672 at concentrations <1 mM. Because a maximum response to BRL-47672 was not determined,

an EC_{50} value for the drug could not be estimated. Nevertheless, based on the K_D of BRL-47672 determined in *experiment 1*, 50% of the response to β_2-adrenoceptor activation would have been expected to occur at a concentration no higher than 16 μM. At this concentration no cAMP response was observed, and so these results are consistent with the lack of any β_2-adrenoceptor stimulation by BRL-47672. We propose that the increase in cAMP output seen at 1 mM BRL-47672 represents the same nonspecific effect seen in response to clenbuterol.

Second-messenger studies in vivo (expts 3 and 4). In *experiment 3* there were no significant increases in cAMP when the mean values were compared at individual time points. However, when the total cAMP output was calculated for each animal by estimating the area under the plot of cAMP concentration vs. time (Fig. 4), it was found that the mean integrated values differed ($P = 0.05$) between control and β-agonist-treated rats. Mean integrated values were not different between groups treated with clenbuterol and BRL-47672, and judging from Fig. 4, there was no apparent difference in the rate of onset of action of the two drugs. The maximum difference in cAMP output between drug-treated and control rats was observed between 45 and 120 min after agonist injection. In *experiment 4* the mean cAMP output from rats treated with BRL-47672 was numerically higher than in control rats 90 min after agonist injection, and the difference between these two groups was similar in magnitude to that observed in *experiment 3* (Fig. 5). However, in *experiment 4* the rats showed an unexpectedly high degree of variation in response, with the result that no statistically significant differences between treatment groups could be detected.

Growth experiments (expts 5, 6, and 7). The results of the three growth experiments are summarized in Fig. 6. The effects of BRL-47672 compared with placebo treatment were as follows. The rate of weight gain was

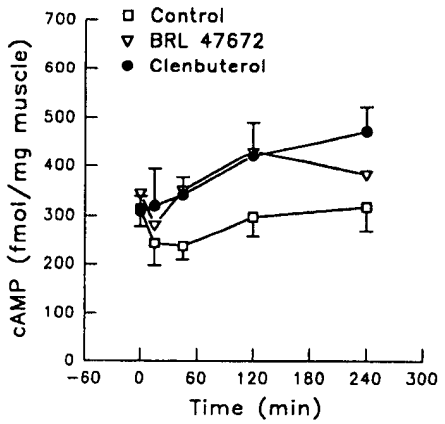


Fig. 4. Time course of drug-induced increase in cAMP concentration determined in biopsy samples of rat skeletal muscle (*expt 3*). Rats were anesthetized using ketamine and xylazine, and then they were given intraperitoneal injections of saline, BRL-47672 (40 μg/rat), or clenbuterol (40 μg/rat). Samples of muscle were removed at time intervals shown and then snap-frozen in liquid N. Each point represents mean ± SE of 6 observations. SE values have been omitted from points for BRL-47672-treated rats for sake of clarity but were not different from those observed for clenbuterol-treated animals.

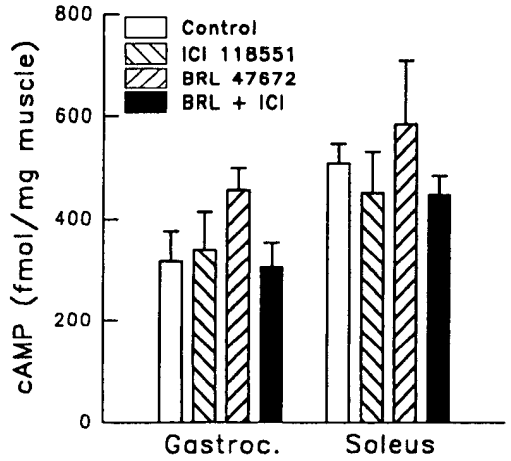


Fig. 5. Concentrations of cAMP in gastrocnemius (gastroc.) and soleus muscles of rat (*expt 4*). Animals were anesthetized using ketamine and xylazine and were given injections of β_2-adrenoceptor selective antagonist ICI-118551 (ICI) or saline as indicated in key. After 15 min rats received second injection of either saline or BRL-47672 (BRL) as indicated. Samples of muscle were removed 90 min later and snap-frozen in liquid N. Each point represents mean ± SE of 5 observations. There were no significant differences between treatment groups (analysis of variance, ANOVA).

increased markedly and to a similar extent in both *experiments 5* and *6* (69–74%, $P < 0.01$). There was no effect on feed intake, and hence the increased growth rate resulted from improved feed conversion efficiency ($P < 0.01$). Because of the short duration of the treatment periods, the mean net difference in body weight between control and treated rats at the end of the experiments was only 10.9 and 7.4 g in *experiments 5* and *6*, respectively. These body weight changes were reflected by increased carcass weight: 9.9 and 7.6 g in *experiments 5* and *6*, respectively. The weight of the gastrocnemius muscle was increased by BRL-47672 in both experiments; a 19% increase was observed in *experiment 5* after 6 days of treatment ($P < 0.01$), and a 6.6% increase was seen in *experiment 6* after 4 days of treatment ($P < 0.05$). Soleus muscle mass was not increased in either experiment. There was a tendency for BRL-47672 to increase the mass of the heart, with a 9.4% increase seen in *experiment 5* ($P < 0.1$) but a smaller, nonsignificant increase (4.5%) in *experiment 6*. There were no significant effects of BRL-47672 on the mass of the periuterine fat pad, although the variance observed for this parameter was particularly large.

When the β_1-adrenoceptor antagonist CGP-20712A was given alone (*expt 7*) there was no significant effect on any of the parameters measured, other than a small increase in feed intake ($P < 0.05$). In addition, CGP-20712A failed to attenuate any of the responses seen to BRL-47672 (*expt 6*).

When the β_2-antagonist ICI-118551 was given alone (*expt 5*) there were no significant effects when compared with control rats. However, an effect on receptor density was apparent when ICI-118551- and BRL-47672-treated rats were compared. In *experiment 5* the density of β_2-adrenoceptors in gastrocnemius muscle membranes from control rats was 103 ± 9.6 fmol/mg protein. The density of β_2-adrenoceptors tended to be increased

by the β_2-antagonist ICI-118551 [maximal binding capacity (B_{max}) 145 ± 21.3 fmol/mg protein], whereas the putative β_2-agonist BRL-47672 tended to have the opposite effect (B_{max} 63.9 ± 7.75 fmol/mg protein), resulting in a significant difference in receptor density between the agonist- and antagonist-treated animals ($P < 0.05$).

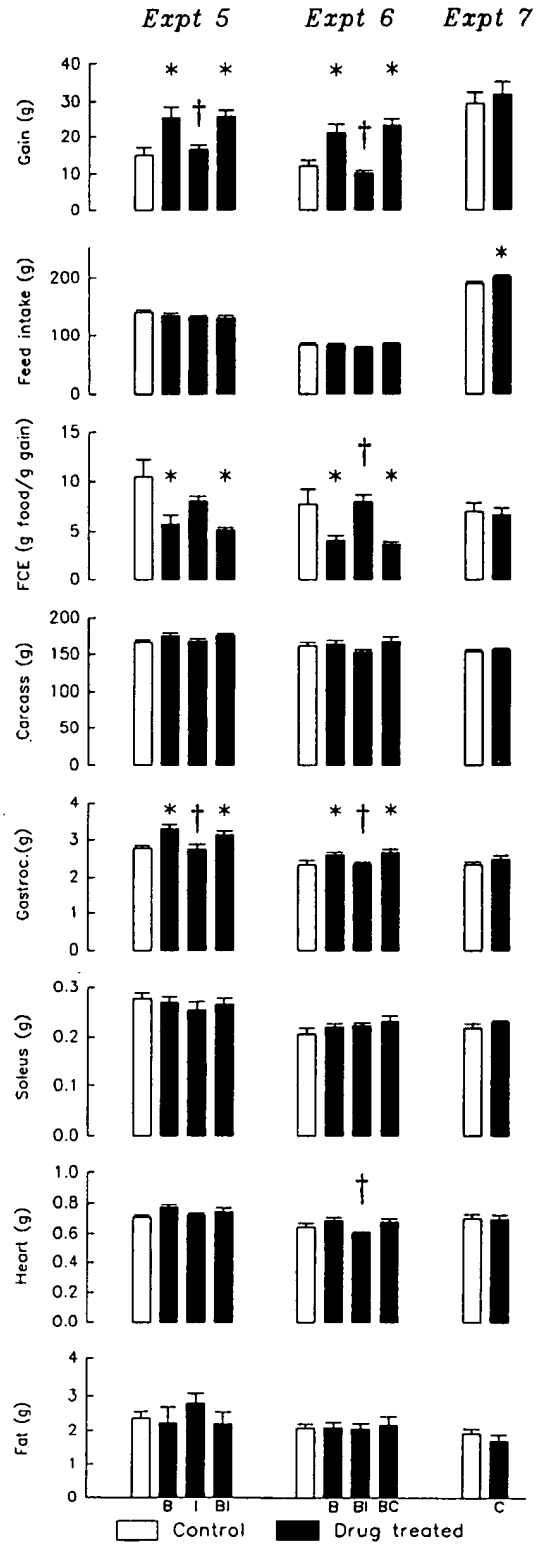


Fig. 6. Effects on growth of putative β_2-adrenoceptor agonist BRL-47672 (B), β_2-adrenoceptor-selective antagonist ICI-118551 (I), and β_1-selective antagonist CGP-20712A (C). These drugs were given alone or in combination to female rats in 3 experiments that lasted for 6, 4, and 10 days (*expts 5, 6,* and *7,* respectively). Values presented for weight gain and feed intake are for duration of each experiment; tissue weights were recorded on last day of each experiment after animals had been killed. BRL-47672 was administered in diet (5 mg/kg feed) as was CGP-20712A (200 mg/kg feed). In *experiment 6* ICI-118551 was also administered in diet (200 mg/kg feed), but in *experiment 5* antagonist was given twice daily by subcutaneous injection (1 mg/rat). FCE, feed conversion efficiency. Results shown are means ± SE for 6 rats (*expts 5* and *7*) or 5 rats per treatment (*expt 6*). Within each experiment means were compared using ANOVA and resultant P values are presented in text. *Means that differ from control values. †Means that differ from values for treatment with BRL-47672 alone. Means separation was achieved using Student's t-test (*expt 7*) or Tukey's test (*expts 5* and *6*) with α-level of 0.05, except for gastrocnemius muscle in *experiment 6* ($\alpha = 0.1$).

The receptor density in animals given the combined treatment was strikingly similar to that of control rats (B_{max} 101 ± 8.4 fmol/mg protein), suggesting that the two ligands were present in skeletal muscle at concentrations sufficient to counteract one another. However, in this experiment subcutaneous injections of ICI-118551 failed to attenuate the effects of BRL-47672 on growth rate or on gastrocnemius muscle mass.

In contrast to the results of *experiment 5,* oral treatment with ICI-118551 at a higher dose (*expt 6*) completely abolished the weight gain in response to BRL-47672 ($P < 0.01$) with a similar antagonism of the effects on heart and gastrocnemius muscle ($P < 0.1$). In this experiment the effects of ICI-118551 were in contrast to those of the β_1-antagonist CGP-20712A, which did not attenuate these responses.

DISCUSSION

The aim of this study was to test the hypothesis that BRL-47672 causes its anabolic effects in rats by interacting either directly or indirectly with β_2-adrenoceptors. The muscle membrane preparation used to examine the binding of BRL-47672 has been shown previously to contain a predominant population of β_2-adrenoceptors (26), and the affinities determined in the present study for clenbuterol and (−)-isoproterenol are in close agreement with values published previously for rat-lung and human β_2-adrenoceptors (13). The K_D value determined for BRL-47672 was low in comparison with the values for clenbuterol and (−)-isoproterenol, but these data alone do not preclude the possibility that BRL-47672 can act via β_2-adrenoceptors, because other selective β_2-agonists such as salbutamol, and the endogenous β_2-agonist epinephrine, have similarly low binding affinities (2.4 and 10.5 μM, respectively; C. A. Javro, G. G. Pegg, and M. N. Sillence, unpublished data). However, although BRL-47672 was shown to be able to bind to β_2-adrenoceptors, the adenylyl cyclase response to this compound seen in vitro was not consistent with β_2-adrenoceptor activation, and in any case was observed only at millimolar concentrations, which would be difficult to achieve in vivo. From this observation we rea-

soned that the anabolic effects of BRL-47672 are unlikely to be mediated by a direct action of the unmetabolized drug on β_2-adrenoceptors, a conclusion which is in accordance with the findings of Arch et al. (2). The relatively weak action of clenbuterol in stimulating cAMP production in vitro is surprising, in view of the drug's potency for increasing skeletal muscle growth. Nevertheless, these data are consistent with those from previous studies, which have shown that clenbuterol is only a partial agonist at β_2-adrenoceptors (20).

Although BRL-47672 was shown to be a weak activator of adenylyl cyclase in vitro, our experiments using anesthetized rats showed that the drug caused a rapid increase in muscle cAMP content in vivo, similar to the response observed with the β_2-adrenoceptor agonist clenbuterol. This result suggests that a receptor in muscle, which is linked to cAMP, can be stimulated by BRL-47672 indirectly, either after rapid metabolism of the drug to a compound that activates β-adrenoceptors or via the release of a secondary endogenous mediator. The predominant cAMP-linked receptor in rat gastrocnemius muscle is the β_2-adrenoceptor (25, 26, 28), and the endogenous agonists for this receptor are epinephrine and norepinephrine. However, because epinephrine and norepinephrine infusions do not cause growth stimulation (22), it is unlikely that the release of either catecholamine mediates the anabolic effects of BRL-47672. Therefore, in *experiments 5* and *6* we focused on the hypothesis that BRL-47672 is metabolized in vivo to a β_2-adrenoceptor agonist.

In growth studies using BRL-47672 we detected no change in food intake but observed an increased rate of weight gain. This was accounted for by accelerated muscle growth, with the gastrocnemius muscle showing a particularly large anabolic response. All of these effects are characteristic responses to β_2-adrenoceptor agonists such as clenbuterol (5, 23, 26). It is also well recognized that clenbuterol and other β_2-agonists cause β_2-adrenoceptors to downregulate (9, 25, 26). In *experiment 5* BRL-47672 caused an effect on skeletal muscle β_2-adrenoceptor density, which was similar to that seen in earlier studies of clenbuterol (26) and opposite to that seen with the β_2-antagonist ICI-118551.

In a previous study we confirmed that the anabolic effects of clenbuterol were β_2-adrenoceptor-mediated, by demonstrating that these effects could be reduced by twice-daily injections of the β_2-antagonist ICI-118551 for 10 days (26). A similar protocol was followed in *experiment 5,* in which ICI-118551 failed to block the growth response to BRL-47672. Initially, we interpreted these results as showing that the effects of BRL-47672 were not, in fact, β_2-adrenoceptor mediated (14). However, on reexamining the results of our previous study we discovered that although the response to clenbuterol was almost abolished by ICI-118551 after 10 days of treatment, the antagonist had no apparent effect on weight gain for the first 5 days of the experiment and little effect by *day 6.* In the present study *experiment 5* was unfortunately terminated on *day 6,* because our supply of ICI-118551 was limited. Together, these results show that as well as being dependent on dose, the effects of ICI-118551 are also dependent on the duration of treatment. The reason for this is difficult to explain but should be examined in terms of the "physiological balance" between the actions of the agonist and antagonist. We believe that the antagonist ICI-118551 has a relatively short plasma half-life and that it is unlikely that the delayed effect was due to the steady accumulation of ICI-118551 in the plasma over several days, until a threshold concentration was reached. Instead, in the earlier study the balance was probably altered in favor of ICI-118551 because of the fact that the anabolic effects of clenbuterol attenuate after repeated dosing.

A different protocol for examining the effects of combined clenbuterol and ICI-118551 treatment was employed by Choo et al. (5), who administered both drugs in the diet over 4 days, with the antagonist given at a dose of 200 mg/kg feed. In *experiment 6,* when ICI-118551 was administered according to the same protocol, the weight gain response to BRL-47672 was prevented, as were the modest changes seen in the mass of the heart and gastrocnemius muscle. The difference in activity observed when the drug was given by injection or feeding may partly reflect the fact that the drug was used at a higher dose in *experiment 6* than in *experiment 5.* It is also probable that ICI-118551 has a shorter plasma half-life than clenbuterol. Thus ingestion of the antagonist in the feed, taken over several small meals, would have resulted in a more even plasma concentration than that achieved by twice-daily injection. It seems that the route of administration not only has a marked effect on the anabolic activity of β_2-agonists (5) but also on the anti-anabolic effect of short-acting antagonists.

The drug ICI-118551 has not been reported to bind to receptors other than β-adrenoceptors, and it is known to selectively block the β_2-adrenoceptor subtype with a binding affinity that is 50 to 100-fold higher than that for the β_1- and β_3-subtypes (7, 16). Thus, together with our other findings, the results of *experiment 6* are consistent with the hypothesis that β_2-adrenoceptors mediate the anabolic effects of a BRL-47672 metabolite. Despite leading us to this conclusion, the present results do not explain why there was not a perfect inverse correlation between the drug-induced effects on muscle growth and their actions on β_2-adrenoceptor density in *experiment 5.* The relationship between muscle growth and the downregulation response of β_2-adrenoceptors in these tissues has been studied previously using the β_2-agonist cimaterol (9). These workers reported that the muscle growth response to cimaterol became attenuated after 14 days of treatment, but that a significant reduction in β_2-adrenoceptor density preceded this, occurring after only 3 days of treatment. Furthermore, in our own recent experiments using a different β-agonist, BRL-26830, (N. G. Moore, G. G. Pegg, and M. N. Sillence, unpublished data), we found that the downregulation of β_2-adrenoceptors in gastrocnemius muscle after 10 days of treatment occurred at lower doses than those required to cause a measurable increase in growth. Thus it appears that β_2-adrenoceptor density is a more rapid and sensitive indicator of β_2-adrenoceptor activa-

tion or blockade than is muscle growth. These results do not suggest to us, however, that β_2-adrenoceptor activation and muscle growth are unrelated.

Because both β_1- and β_3-adrenoceptor subtypes have also been reported to be present in some rat muscles (3, 25) and because ICI-118551 will block these receptors at sufficiently high concentrations, an additional treatment group was employed in *experiment 6* to investigate the effects of CGP-20712A. This antagonist has a high affinity for β_1-adrenoceptors (K_D 1–3 nM; Refs. 7 and 16) and an affinity for β_2-adrenoceptors that is 2,000–7,000 times lower (K_D 5–7 μM; Refs. 7 and 16). CGP-20712A and ICI-118551 block β_3-adrenoceptors with equally low affinity (K_D 6.3 and 2.5 μM, respectively; Ref. 7). Therefore, if the anabolic effects of BRL-47672 had occurred as a consequence of β_1-adrenoceptor stimulation, then CGP-20712A would have been a more effective antagonist than ICI-118551. Conversely, if β_3-adrenoceptors were the principal subtype involved in the growth response, then ICI-118551 and CGP-20712A would be equally effective or ineffective as antagonists, assuming no major differences in the rate of absorption or metabolism of these two compounds. Unfortunately we have no pharmacokinetic data for CGP-20712A in the rat. Nevertheless, with the latter assumption in mind, the finding that CGP-20712A caused no attenuation of the effects of BRL-47672 is consistent with the lack of any contribution to the growth response from β_1- or β_3-adrenoceptor activation.

In summary, our results support the conclusion that the anabolic effects of BRL-47672 are dependent on the activation of β_2-adrenoceptors. However, the drug does not appear to activate these receptors directly in vitro, and this suggests the possibility that BRL-47672 is metabolized in vivo to a potent β_2-adrenoceptor agonist.

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The Effect of the β_2-Adrenoceptor Agonist Prodrug BRL-47672 on Cardiovascular Function, Skeletal Muscle Myosin Heavy Chain, and MyoD Expression in the Rat

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ABSTRACT

The intracellular mechanisms that regulate changes in postnatal myosin heavy chain (MHC) expression are not well established. The major objective of this study was to examine the acute and chronic effects of administration of BRL-47672, the prodrug of the β_2-adrenoceptor agonist clenbuterol on MHC and MyoD transcription factor expression to determine whether or not changes in MHC composition are preceded by changes in MyoD protein expression. To assess to what extent the use of BRL-47672 minimized cardiovascular effects, its hemodynamic actions were compared with those of clenbuterol. The effect of BRL-47672 on heart rate, mean arterial blood pressure, and hindquarters vascular conductance was significantly less than that of clenbuterol after a single i.p. injection (250 μg kg^{-1} body mass). In the main study, 4-week old rats were given BRL-47672 (900 μg kg^{-1} body mass) or an equivalent volume of saline (control) daily for 1, 28, or 56 days. Soleus muscle (SOL) was excised and MHC and MyoD expression analyzed. After 4 weeks, SOL from the BRL-47672-treated animals had significantly faster MHC composition (49 ± 2% MHCIIA) compared with those from the control animal (39 ± 3% MHCIIA, $P < 0.05$). MyoD expression increased by 40% after 1 day of BRL-47672 administration ($P < 0.05$) before a change in MHC composition. In conclusion, these data suggest that increased expression of fast-type MHCIIA expression in rat SOL induced by BRL-47672 administration is preceded by changes in the level of MyoD transcription factor expression.

The diversity of skeletal muscle fiber types, defined by the expression of particular myosin heavy chain (MHC) isoforms, is believed to be due to a combination of distinct myoblast lineage and extrinsic factors (DiMario and Stockdale, 1997). Postnatally, however, exogenous factors such as innervation, neuromuscular activity, and hormone levels (e.g., thyroid hormone) become the main determinants of fiber type (Termin and Pette, 1992), and fiber type transitions can occur due to altered expression of MHC isoforms (Maltin et al., 1989). Transitions of fiber type in adult skeletal muscle have also been reported in humans during aging (Balagopal et al., 2001), where the decline in both number and size of fast fiber types could contribute to the impaired muscle function reported in the elderly population (Dutta et al., 1997), and in disease states such as chronic obstructive pulmonary disease and heart failure (Drexler et al., 1992; Maltais et al., 1999).

Understanding of the determination and differentiation of muscle fiber type has been advanced with the discovery of a family of myogenic regulatory factors (MRFs), namely, MyoD, myogenin, myf5, and MRF4. These transcription factors share 80% homology within a basic helix-loop-helix motif (Murre et al., 1989) that mediates dimerization and DNA binding to the E-box consensus sequence (CANNTG) found in the control regions of muscle-specific genes, including the MHCIIB gene (Wheeler et al., 1999). It has been proposed that these myogenic factors are essential for development and differentiation of skeletal muscle from myoblast cells (Weintraub, 1993; Olson and Klein, 1994). Knockout studies in mice have shown that during murine embryogenesis, MyoD, myf5, myogenin, and MRF4 are expressed in overlap-

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ABBREVIATIONS: MHC, myosin heavy chain; MRF, myogenic regulatory factor; SOL, soleus muscle; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; ECL, enhanced chemiluminescence; ANOVA, analysis of variance; ICI-118551, (±)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol; CGP-20712A, ([2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulfonate.

ping but distinct patterns, with Myf5 and MyoD acting early to establish myoblast lineage and myogenin acting later to control differentiation (Rawls et al., 1998). After birth, myogenic transcription factor expression decreases, but low levels of expression do persist in adult tissue (Hughes et al., 1993), indicating a possible role in maintaining MHC phenotype and muscle remodeling in adult tissue. Indeed, it has been shown that during development in rat skeletal muscle, MyoD and myogenin accumulate differentially in fast and slow muscles (Hughes et al., 1997), suggesting that MyoD and myogenin may regulate fast and slow myosin expression, respectively. However, it has been shown previously that overexpression of myogenin in transgenic mice resulted in an increase in oxidative enzymes but no change in MHC composition (Hughes et al., 1999). Therefore, it would appear that MyoD expression is a more likely determinant of MHC phenotype than myogenin.

One tool that has been used to study the regulation of skeletal muscle fiber type is the administration of β_2-adrenoceptor agonists (β_2-agonists) such as clenbuterol that, when given chronically, have been reported to induce slow-type I to fast-type II fiber transitions (Zeman et al., 1988), possibly by signaling through MyoD since changes in muscle fiber type have been associated with altered levels of MyoD expression (Hughes et al., 1993; Goblet and Whalen, 1995; Kraus and Pette, 1997). Since MyoD expression has been reported to be higher in fast muscles than in slow muscles (Hughes et al., 1997; Sakuma et al., 1999), it is not known whether these changes in expression of MyoD following fiber type transitions are a consequence or cause of change in MHC phenotype. Furthermore, although administration of clenbuterol is known to bring about slow to fast fiber type transitions, it also causes substantial hemodynamic changes (Rothwell et al., 1987), which might preclude its use in a clinical setting. Theoretically, one way of reducing these undesirable effects would be to administer a prodrug that is metabolized to a β_2-agonist in vivo (Sillence et al., 1995), but it is not known if such a strategy would also reduce the phenotypic effects. BRL-47672 has a chemical structure similar to the β_2-agonist clenbuterol (Fig. 1) but has little direct action on β_2-adrenoceptors. The work of Sillence et al. (1995) demonstrated that, in vitro, BRL-47672 had a low affinity for rat β_2-adrenoceptors compared with clenbuterol and was a poor activator of rat adenylyl cyclase activity in rat skeletal muscle. Conversely, acute administration in vivo resulted in increased adenylyl cyclase activation and, over 6 days of administration, an increase in skeletal muscle mass. Furthermore, these anabolic effects of BRL-47672 were not blocked by daily injection of the β_2-adrenoceptor-selective antagonist ICI-118551 but were blocked when ICI-118551 was administered in the diet. The addition of the β_1-adrenoceptor-selective antagonist CGP-20712A to the diet failed to dampen the anabolic effects of BRL-47672. This led us to conclude that BRL-47672 has little direct action on β_2-adrenoceptors per se but is metabolized rapidly in vivo to a potent β_2-agonist. Finally, Sillence et al. (1995) claimed that BRL-47672 was a less potent stimulator of heart rate than clenbuterol, which seems logical given its reported lack of direct effect on β_2-adrenoceptors. On the basis of the observations of Sillence et al. (1995), it is not unreasonable to infer that chronic administration of BRL-47672 would influence cell signaling pathways associated with skeletal muscle mass and fiber type in the rat in a manner similar to clenbuterol while having a comparatively attenuated hemodynamic effect.

The first objective, therefore, of the present work was to compare the in vivo hemodynamic effects of clenbuterol and BRL-47672 in conscious rats. The second objective was to examine the acute and chronic effects of BRL-47672 administration on MHC and MyoD transcription factor expression to determine whether or not β_2-agonist-induced changes in MHC composition are preceded by changes in MyoD protein expression. Knowledge of the intracellular regulatory factors that underlie β_2-agonist-induced changes in postnatal MHC expression would aid an understanding of how skeletal muscle adaptations occur, not only during normal development but also in disease states that lead to muscle dysfunction. This information could be of considerable clinical importance if, for example, selective fast fiber type atrophy causing debilitating muscular dysfunction could be halted or even reversed by administration of a β_2-agonist compound that promotes fast-type myosin expression.

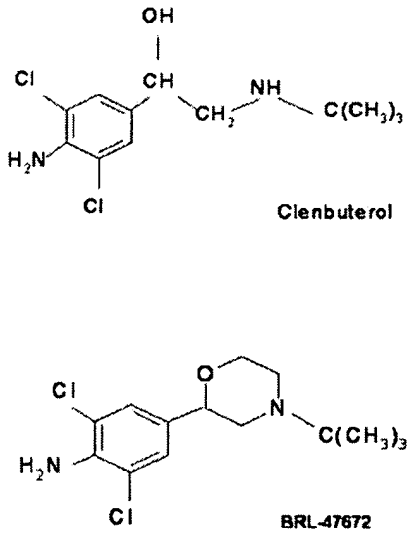


Fig. 1. Structural properties of clenbuterol and BRL-47672 (Sillence et al., 1995).

Materials and Methods

Cardiovascular Studies

Cardiovascular measurements were performed on male Sprague-Dawley rats (n = 8) weighing between 420 and 470 g. Surgery was performed under general anesthesia (fentanyl; Janssen-Cilag Ltd., High Wycombe, UK) and medetomidine (Pfizer Limited, Sandwich, UK), 300 $\mu g\ kg^{-1}$ of each, i.p., reversed with nalbuphine (Bristol-Myers Squibb, Hounslow, UK) and atipamezole (Pfizer), 1 $mg\ kg^{-1}$ of each, s.c., as described previously (Gardiner et al., 2002). Catheters were placed in the distal abdominal aorta (via the caudal artery) to monitor arterial blood pressure and heart rate and also in the peritoneum for drug administration. Hindquarters blood flow was monitored by a pulsed Doppler probe implanted around the distal abdominal aorta.

Animals were administered either clenbuterol (n = 8) or the BRL-47672 (n = 8) at 250 $\mu g\ kg^{-1}$ i.p., which is equivalent to molar concentrations of 0.80 and 0.82 μM, respectively. Continuous recordings of cardiovascular variables were made using a customized computer-based system [Hemodynamics Data Acquisition System

(HDAS), University of Limberg, Maastricht, The Netherlands] connected to the Gould transducer amplifier (model 13-4615-50; Gould Instrument Systems Inc., Cleveland, OH). Raw data were collected every 2 min and stored to disc at 5-s intervals for up to 40 min after drug administration. Within-group analysis was conducted using Friedman's test (Theodorsson-Norheim, 1987), and between-group comparisons were made with the Mann-Whitney U test applied to areas under curves. A P value of <0.05 was taken as significant.

Muscle Studies

Dose-Dependent Effects of BRL-47672. Six-week-old male Wistar rats were given daily subcutaneous injections of either 250, 450, or 900 $\mu g\ kg^{-1}$ body mass of BRL-47672 ($n = 4$) or an equivalent volume of saline ($n = 4$) for 8 weeks. On the day after the final injection, rats were anesthetized with thiobutabarbital sodium (Inactin, 120 mg kg^{-1}, i.p.) and SOL of the hind limb excised, snap frozen in liquid nitrogen, and then stored at −80°C for subsequent analysis. Given that the SOL muscle is known to contain only type I and type IIA myosin, the gastrocnemius muscle of the saline-treated animals was also excised to best illustrate the separation of MHCs using SDS-PAGE (6%) gels.

Time-Dependent Effects of BRL-47672. Four-week-old male Wistar rats were given daily subcutaneous injections of 900 $\mu g\ kg^{-1}$ body mass of BRL-47672 or saline (control) for 1 day ($n = 10$), 4 weeks ($n = 10$), or 8 weeks ($n = 10$). The day after the final injection, rats were anesthetized (Inactin, 120 mg kg^{-1}, i.p.), and the SOL was excised and frozen as described above.

All animals were kept under 12-h light/dark cycles and food and water was provided ad libitum. All work was conducted in accordance with the Home Office Scientific Animals (Scientific Procedures) Act 1986, and the animals were killed humanely at the end of the experiment.

Myosin Heavy Chain Analysis. Crude myosin was prepared as described previously (Termin et al., 1989). In brief, SOL (approximately 50 mg) was homogenized in 10 vol of buffer (0.3 M KCl, 0.1 M KH_2PO_4, 50 mM K_2HPO_4, 10 mM EDTA, and 5 mM DTT, pH 6.5) containing 10 $\mu g\ ml^{-1}$ 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 0.01 $\mu g\ ml^{-1}$ pepstatin A, and 0.1 $\mu g\ ml^{-1}$ leupeptin. Muscle homogenate was then left on ice for 15 min with periodic vortexing to facilitate protein extraction. Samples were then centrifuged at 6000g for 15 min, and the supernatant was mixed 1:1 with glycerol before being stored at −20°C. Supernatant total protein was determined (Bradford, 1976), and MHCs (MHCI and MHCIIA) were electrophoretically separated by applying between 0.1 and 0.5 μg of protein extract to 0.75-mm thick SDS-PAGE (6%) gels containing 40% glycerol. Electrophoresis was run at 70 V for 17 to 18 h, and the gels were then silver-stained using Bio-Rad Silver-Stain Plus kit (Bio-Rad, Hemel Hempstead, UK).

Histochemistry. To determine whether SDS-PAGE analysis of the MHCs was a reflection of fiber type composition rather than fiber hypertrophy, SOL from 8-week BRL-47672-treated and control animals ($n = 5$ in each) was subjected to histochemical analysis. Serial cross-sections (20 μm thick) were cut on a cryostat at −20°C and stained for myofibrillar ATPase after prior preincubation of sections at pH 4.2 and 10.3 (Brooke and Kaiser, 1970). Acid preincubation consisted of 5 min in 142 mM barbital acetate buffer, whereas alkaline preincubation consisted of 20 min in 36 mM $CaCl_2$, buffered with 100 mM Tris. Sections were then incubated at room temperature for 45 min in 20 mM sodium barbital, 18 mM $CaCl_2$, and 4.5 mM ATP (pH 9.4).

MyoD Analysis. Frozen samples of SOL were homogenized in buffer (20 mM Tris, 5 mM EDTA, and 5 mM DTT, pH 7.5) containing 300 mM NaCl to extract nuclear protein. Protease inhibitors were added as for MHC analysis. Homogenates were left on ice for 30 min with periodic vortexing to facilitate nuclear protein extraction before being centrifuged at 10,000g for 20 min. Supernatant protein content was measured (Bradford, 1976), and an aliquot of supernatant was then mixed 1:1 with 2× SDS mix. Protein extracts (100 μg) were analyzed by 10% SDS-PAGE gels. Separated protein was electrotransferred onto Hybond ECL membrane (Amersham Biosciences UK, Ltd., Little Chalfont, UK) at 200 mA for 2 h (Towbin et al., 1979) and then blots were probed with a commercially available MyoD (M-318; 1:1000) polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and visualized using enhanced chemiluminescence (ECL; Amersham Biosciences UK, Ltd.). To confirm equal protein loading, immunoblots were re-probed with a monoclonal sarcomeric α-actinin antibody (Sigma-Aldrich, St. Louis, MO) at a dilution of 1:1000.

Preparation of Nuclei from Skeletal Muscle. To confirm that the MyoD antibody was detecting a nuclear protein on the immunoblots of the supernatant protein extracts, nuclei from skeletal muscle were prepared by centrifugation through high sucrose gradients (Koffer and Brownson, 1982). Fresh SOL (100 mg) was diced and gently homogenized using a Kontes glass homogenizer in 10 volumes of ice-cold TM buffer (10 mM Tris, 5 mM $MgCl_2$, pH 7.6) containing 0.25 M sucrose, 2 mM DTT, and a cocktail of protease inhibitors (Sigma-Aldrich). The homogenate was then filtered through gauze, and the filtrate was centrifuged at 2500g for 15 min at 4°C. The sucrose supernatant was then discarded, and the pellet was homogenized in 1 ml of TTM buffer (TM buffer containing 0.5% Triton) with 0.25 M sucrose for 5 min before being centrifuged at 2500g for 15 min at 4°C. The sucrose supernatant was discarded, and the pellet was homogenized in 0.5 ml of 2.4 M sucrose in TM buffer and centrifuged at 35,000g for 1 h at 4°C to pellet the nuclei. The nuclear pellet was then resuspended in 70 μl of TM buffer and stored at −80°C. Twenty microliters were then mixed with an equal volume of 2× SDS mix and run on 10% SDS-PAGE and MyoD protein detected, as described for whole muscle analysis.

Quantification and Statistical Analysis. Silver-stained gels and immunoblots were scanned, and the densitometric intensity of the bands was analyzed using the OPTIMAS software package (Media Cybernetics, Inc., Silver Spring, MD). The ECL signal was in the

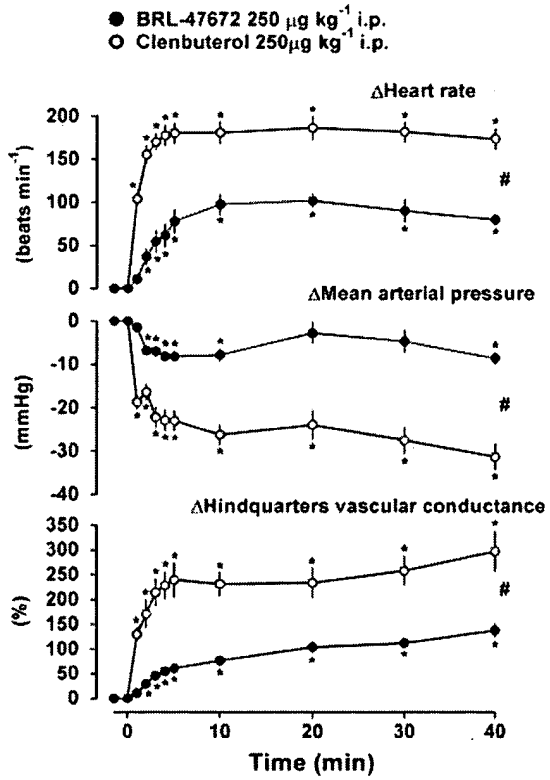


Fig. 2. Cardiovascular responses to clenbuterol or BRL-47672. Cardiovascular responses to clenbuterol or BRL-47672 (250 $\mu g\ kg^{-1}$) in conscious Sprague-Dawley rats ($n = 8$). Values represent mean, and the vertical bars show S.E.M. *, $P < 0.05$ versus baseline within group (Friedman's test). #, $P < 0.05$ BRL-47672 versus clenbuterol (Mann-Whitney U test applied to areas under curves).

linear range, and a standard whole cell lysate, prepared from skeletal muscle, was used to enable blot-to-blot comparisons to be made. Values in text are represented as mean ± S.E.M. ANOVA (one-way, plus least significant difference post hoc test) was used to examine dose-dependent effects of BRL-47672 on MHC expression. ANOVA (two-way) was used to examine time-dependent effects of BRL-47672 on MHC and MyoD expression. Where time and treatment effects were observed, unpaired *t*-tests were used to examine differences between treatment groups and time points. Statistical significance was accepted at the $P < 0.05$ level. The Pearson correlation coefficient was used to describe the linear relationship between the percentage of MHC and percentage of fiber type.

Results

Cardiovascular Studies

Clenbuterol produced significant cardiovascular effects within 5 min of administration, with increased heart rate, reduced mean arterial pressure, and raised vascular conductance in the hindquarters. These effects were sustained over the 40-min recording period. In contrast, the rates of onset and magnitude of the cardiovascular effects following administration of BRL-47672 were significantly lower compared with those observed following clenbuterol administration (Fig. 2).

Muscle Studies

Dose-Dependent Effects of BRL-47672 on MHC Expression. SDS-PAGE resolved two MHCs in SOL, type I and II, with no expression of fast type IIB or IIX myosin in either treatment group (Fig. 3). Densitometric analysis of the MHC silver-stained gels showed that after 8 weeks, SOL from control animals was composed of 83 ± 3% MHCI and 17 ± 3% MHCIIA (Fig. 4, a and b). Administration of BRL-47672 at 250 $\mu g\ kg^{-1}$ increased the proportion of MHCIIA by 15% compared with control, whereas the 450 and 900 $\mu g\ kg^{-1}$

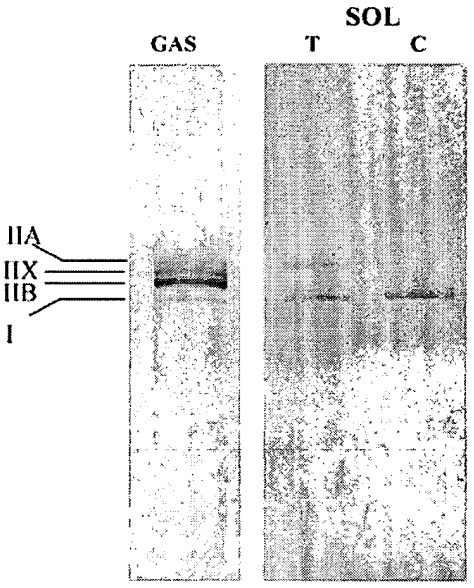


Fig. 3. SDS-PAGE analysis of myosin heavy chains. Myosin heavy chain expression in SOL after 8 weeks of administration of saline control (C) or BRL-47672 (T) compared with rat gastrocnemius (GAS) myosin, analyzed on 6% SDS-PAGE gels. Approximately 0.1 μg of protein was loaded in each lane and the gel run at 70 V for 17 h.

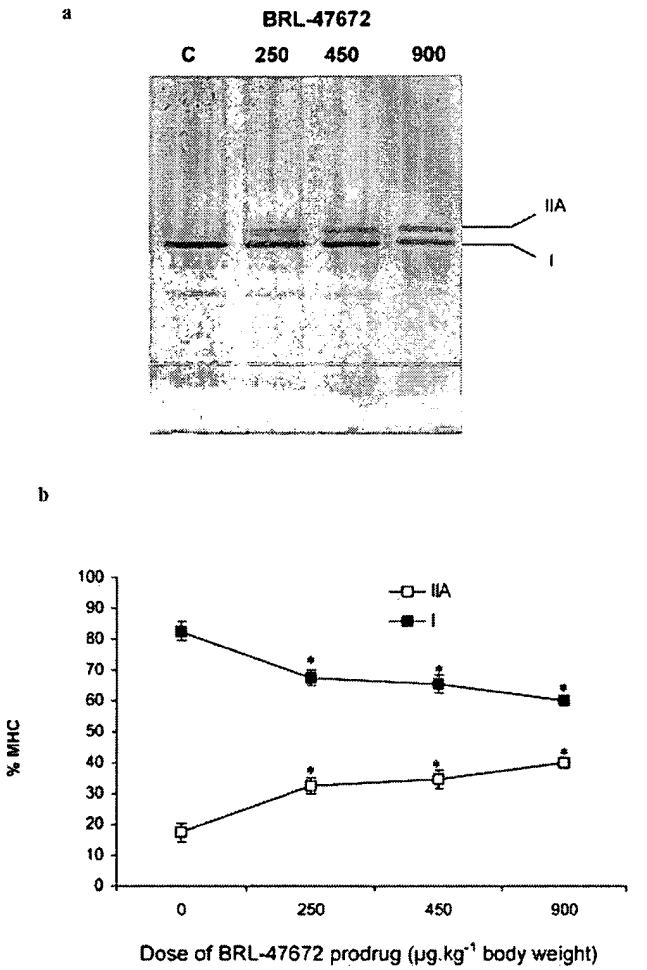


Fig. 4. Dose-dependent effects of BRL-47672 on myosin heavy chain expression. a, representative 6% SDS-PAGE gel of myosin heavy chain expression after 8 weeks of saline control (C) or after 250, 450, or 900 $\mu g\ kg^{-1}$ body mass of BRL-47672. Approximately 0.5 μg of protein were loaded in each lane, and the gel was run at 40 V for 17 h. b, dose-response curve of the effects of BRL-47672 on the percentage of type I and type IIA myosin. Values are mean ($n = 4$) with S.E.M. shown by vertical bars. *, $P < 0.01$ versus control (one-way ANOVA plus least significant difference post hoc test).

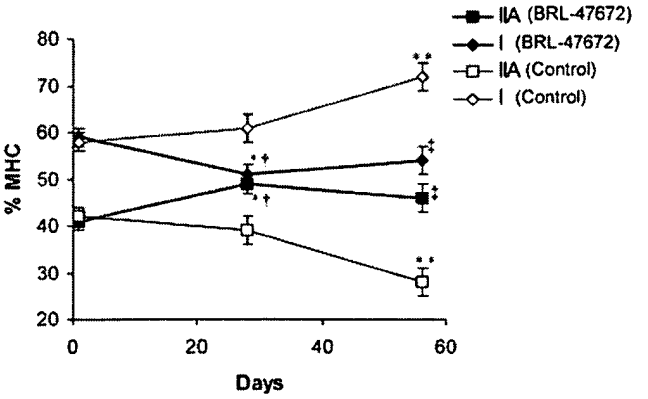


Fig. 5. Time-dependent effects of BRL-47672 on myosin heavy chain expression. Changes in MHC expression after BRL-47672 administration (900 $\mu g\ kg^{-1}$ body mass) or saline for 8 weeks. Values are expressed as mean ± S.E.M. ($n = 10$). *, $P < 0.05$ and **, $P < 0.01$, different from 1-day value within group. ‡, $P < 0.05$ and †, $P < 0.01$, different from corresponding control value (two-way ANOVA).

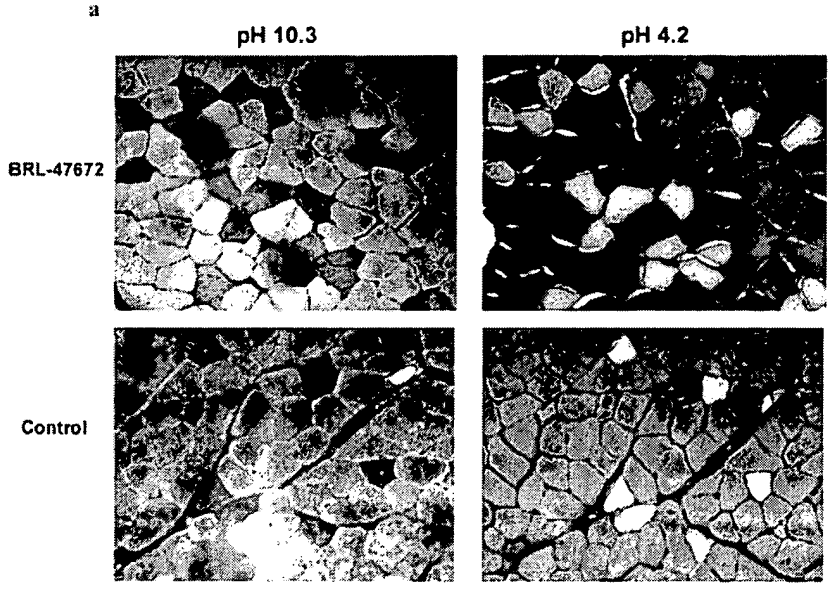
doses increased the proportion of MHCIIA by 18 and 23%, respectively (Fig. 4, a and b).

Time-Dependent Effects of BRL-47672 on MHC Expression. Over the 8-week study, SOL shifted toward a slower more oxidative phenotype. This was seen as an increase in MHCI expression from 58 ± 2% to 72 ± 3% and a corresponding reduction in fast-type MHCIIA expression from 42 ± 2% to 28 ± 3% ($P < 0.01$; Fig. 5). There was no significant difference in MHC expression between the BRL-47672-treated and control group after 1 day of dosing (Fig. 5); however, after 4 weeks of BRL-47672 administration, the proportion of MHCIIA (49 ± 2%) was significantly higher than at the 1-day time point (41 ± 2%; $P < 0.05$) and significantly higher than in the control group at that time (39 ± 3% $P < 0.05$) (Fig. 5). At 8 weeks, the level of MHCIIA expression in the BRL-47672-treated group was not different from the 1-day time point, although compared with the control group, MHCIIA expression was significantly higher ($P < 0.01$) and MHCI significantly lower ($P < 0.01$).

Histochemistry. To determine whether the measurements of MHC reflected fiber type composition, SOL from treated and control animals was subjected to myofibrillar ATPase staining. Histochemical analysis (Fig. 6a) showed that the percentage of type IIA fiber type was significantly higher ($P < 0.01$) in the BRL-47672-treated animals (38 ± 4%, $n = 5$) compared with control (16 ± 3%, $n = 5$). Furthermore, plotting the percentage of fiber type as determined by ATPase staining against the percentage of MHC as determined by SDS-PAGE revealed a strong linear correlation ($r = 0.99$) between fiber type and MHC expression (Fig. 6b).

MyoD Analysis. The cDNA sequence of rat MyoD predicts a protein of 34 kDa molecular weight; however, a number of molecular weights have been reported for MyoD on SDS-PAGE, ranging from 33 to 48 kDa (Hughes et al., 1997; Sakuma et al., 1999; Tamaki et al., 2000), which may, in part, be due to the high number of basic residues in its sequence. Using a commercially available polyclonal MyoD antibody (Santa Cruz Biotechnology, Inc.), a single band of approximately 35 kDa was detected on SDS-PAGE from SOL whole cell salt extracts. A Western blot of nuclei prepared from fresh SOL detected a band of identical mobility (Fig. 7a).

Densitometric analysis of immunoblots revealed that there was no change in MyoD expression within either treatment group with respect to time; however, MyoD protein expres-



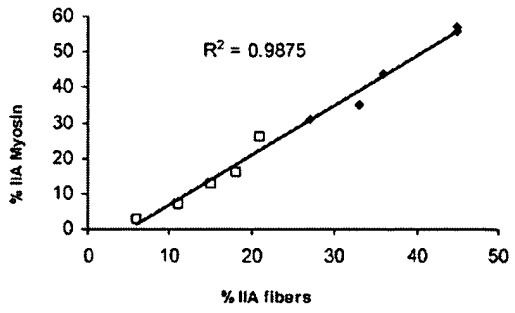


Fig. 6. Correlation of myofibrillar ATPase staining and MHC analysis by SDS-PAGE. a, myofibrillar ATPase staining of SOL cross-sections (20 μm thick) after preincubation at pH 10.3 and 4.2 from 8-week BRL-47672 treatment and from control animals ($n = 5$). b, correlation of percentage of MHCIIA (as determined by SDS-PAGE) against the percentage of type IIA fibers (as determined by histochemical staining). Open boxes, control samples; closed diamonds, BRL-47672-treated samples.

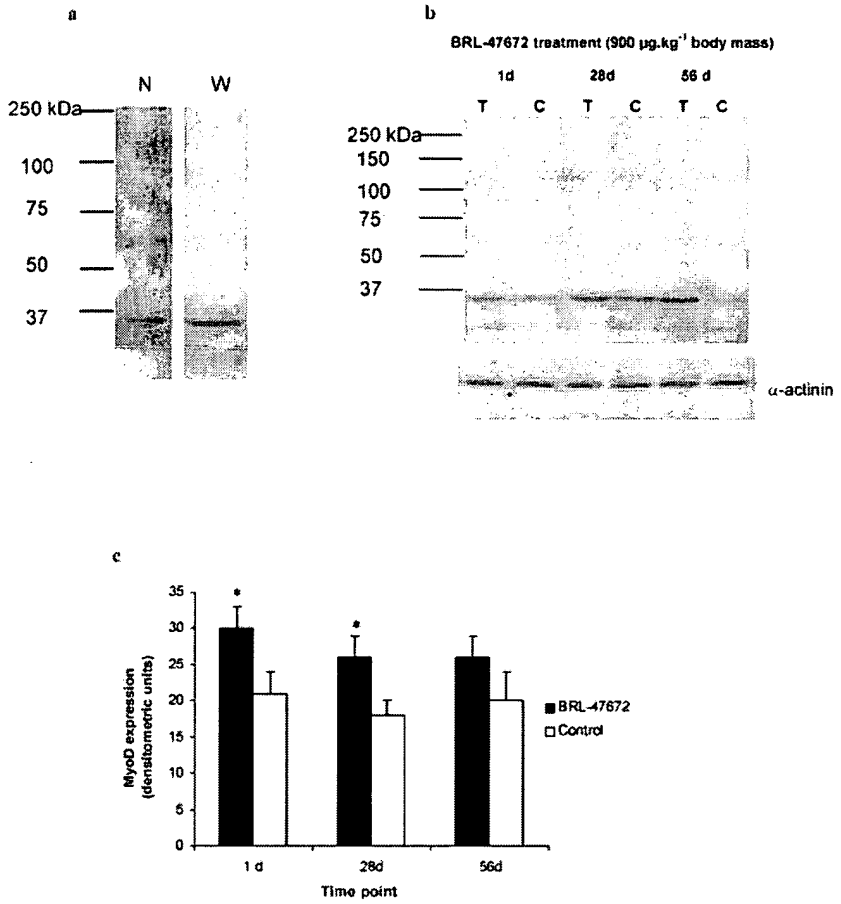


Fig. 7. MyoD analysis. a, immunoblot from 10% SDS-PAGE showing detection of MyoD in SOL nuclei prepared by centrifugation through high-sucrose gradients (N) and in SOL whole muscle extract (W). Blot was probed with MyoD (1:1000) antibody and visualized using ECL. Markers, Bio-Rad precision prestained standards. b, representative immunoblot from SDS-PAGE (10%) analysis of SOL extracts after BRL-47672 treatment (900 $\mu g\ kg^{-1}$ body mass; T) or saline control (C) probed with MyoD (1:1000) antibody and visualized using ECL. Exactly 100 μg of total protein was loaded in each lane. Markers as in panel a. Immunoblot was reprobed with α-actinin (1:1000) to demonstrate equal protein load. c, mean MyoD expression levels in SOL after 1 day, 4 weeks, and 8 weeks of BRL-47672 or saline (control) administration. Values are means ± S.E.M. (n = 10). *, $P < 0.05$ (two-way ANOVA), different from corresponding control value.

sion was significantly higher ($P < 0.05$) in the BRL-47672-treated group (30 ± 3 units, n = 10) compared with control (21 ± 3 units) after 1 day of administration (Fig. 7, b and c). MyoD expression was maintained at a significantly higher level ($P < 0.05$) after 4 weeks in the BRL-47672-treated group (26 ± 3 units) compared with control (18 ± 2 units). By 8 weeks, although MyoD expression was on average higher in the BRL-47672-treated group compared with the control group, this was not significantly different (Fig. 7c).

Discussion

The objective of the present work was to determine whether there were significant differences between the hemodynamic effects of clenbuterol and its prodrug, BRL-47672, and to determine whether or not changes in MHC expression induced by BRL-47672 administration are preceded by changes in MyoD protein expression in rat SOL to ascertain whether MyoD expression plays a role in regulating postnatal MHC composition.

Although clenbuterol is well known to induce slow to fast skeletal muscle fiber type transitions, its substantial cardiovascular actions render it unacceptable for clinical use in conditions where muscle protein accretion and/or remodeling of muscle fiber type might be desirable. Thus, an agent with the anabolic and fiber type remodeling actions of clenbuterol but with lesser cardiovascular effects would be of clinical interest. Since the β_2-adrenoceptors that mediate the effects of clenbuterol on skeletal muscle are pharmacologically indistinguishable from those responsible for its cardiovascular actions (Rothwell et al., 1987), improving target selectivity does not appear to be a likely option. Therefore, the question arises whether or not a prodrug that is metabolized to clenbuterol in vivo would have lesser cardiovascular effects.

Comparing clenbuterol and BRL-47672, it was clear that the effect of the latter on heart rate, mean arterial pressure, and hindquarters vascular conductance were blunted; however, BRL-47672 was at least as potent as clenbuterol at inducing MHC remodeling. Thus, administration of BRL-47672 at a dose of 250 $\mu g\ kg^{-1}$ for 8 weeks induced a 15% increase in MHCIIA expression, although at the higher dose (900 $\mu g\ kg^{-1}$), there was a 23% increase in MHCIIA expression. These changes are comparable with results we have previously published showing that clenbuterol administration (250 $\mu g\ kg^{-1}$ for 8 weeks) increased MHCIIA expression by 13 to 23% (Rajab et al., 2000). Furthermore, it has been reported that rats fed clenbuterol (1.6 mg kg^{-1} for 8 weeks) showed a 23% increase in MHCIIA expression (Zeman et al., 1988), which is similar to the increase in MHCIIA we report here with BRL-47672 but using a considerably lower dose (900 $\mu g\ kg^{-1}$).

To determine whether or not β_2-agonist-induced changes in MHC expression were preceded by up-regulation of MyoD expression, time-dependent changes in MHC and MyoD protein expression were measured. During the 8 weeks of the study, the MHC composition of SOL in the control animals changed, becoming slower because of a transition from MHCIIA to MHCI

(Fig. 5). This pattern of transition, however, did not occur in the SOL of the BRL-47672-treated animals. As a result, the proportion of MHCIIA was significantly higher ($P < 0.01$), and consequently MHCI was significantly lower in the BRL-47672-treated animals compared with control after 4 and 8 weeks.

The strong correlation between the percentage of fiber type and MHC expression (Fig. 6b) clearly indicates that the changes in MHC expression reported here are likely to have been predominantly due to changes in muscle fiber type composition as opposed to hypertrophy of specific fiber types. A change of fiber type with age in rats has been reported previously (Maltin et al., 1989), where the fiber type profile of SOL changed throughout the first year of life. Since the MHC composition of rat muscles in this period is not static, the current data suggest that the effect of BRL-47672 administration was to induce and maintain fast MHCIIA expression in SOL, as opposed to "switching" per se (Fig. 5). Thus, BRL-47672 administration seems to prevent the normal developmental MHC switch from MHCIIA to MHCI in SOL.

Analysis of MyoD protein expression revealed that MyoD expression was significantly increased after 1 day of BRL-47672 treatment (Fig. 7, b and c). These data support previous observations (Hughes et al., 1993) showing that treatment with clenbuterol and thyroid hormone (T_3) resulted in increased MHCIIA and MyoD expression. Notably, however, we report here for the first time that elevation of MyoD protein expression occurred before any change in MHC composition. This suggests that an up-regulation of MyoD is a prerequisite for induction of MHCIIA expression in SOL following β_2-agonist administration.

In the control animals, despite the significant increase in MHCI and reduction in MHCIIA during the 8 weeks (Fig. 5), there was no change in the expression of MyoD with time (Fig. 7c). This suggests that during normal development of SOL, MyoD expression is kept at basal levels and that the increase in MHCI and parallel loss of MHCIIA may be due to maintenance of these low levels of MyoD. If this were the case, the relatively high abundance of the transcription factor, myogenin, which is associated with slow muscle, might be responsible for the increase in type I myosin as the muscle reaches maturation. Indeed, there is evidence to suggest that the expressions of MyoD and myogenin are regulated through independent mechanisms. Thus, in rat, administration of clenbuterol in the diet for 3 days resulted in an increase in MyoD mRNA expression relative to myogenin (Delday and Maltin, 1997). Interestingly, however, clenbuterol, fed to rats with immobilized hind limbs over the same time period, led to a marked increase in myogenin but no change in MyoD. It is worth noting, therefore, that the expression of myogenic transcription factors in response to the administration of β_2-agonists such as clenbuterol could depend on the metabolic status of the muscle.

In summary, our data indicate that induction and maintenance of MHCIIA in SOL is preceded by up-regulation of the transcription factor, MyoD. The finding that BRL-47672 induced MHC remodeling to the same extent as clenbuterol, but with lesser cardiovascular effects, suggests future therapeutic potential for an ultra-slow release formulation in conditions of debilitating fiber type remodeling and selective fiber type atrophy.

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SECONDARY INJURY MECHANISMS IN ACUTE SPINAL CORD INJURY

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☐ **Abstract—Experimental studies in animal spinal cord injury models suggest that preservation of a relatively small number of spinal axons can support neurological recovery. The second National Acute Spinal Cord Injury Study (NASCIS 2) was the first clinical trial to demonstrate that a treatment given after injury can enhance neurological recovery. In this trial, patients treated with high-dose methylprednisolone within 8 hours of spinal cord injury recovered more sensory and motor function than did those treated with placebo. In addition to demonstrating the first effective pharmacological intervention in central nervous system injury, NASCIS 2 identified several critical issues that must be investigated in future preclinical and clinical research. These include drug dose, initiation time, and duration of treatment, as well as combination therapy and injury severity. Addressing these issues systematically will require more reproducible animal models and more accurate outcome measures.**

☐ **Keywords—spinal cord injury; neuroprotection; methylprednisolone; glucocorticoids; preclinical research**

THE SPINAL CORD LESION

Experimental studies of animal spinal cord injury models have played an important role in establishing mechanisms of traumatic tissue damage. In particular, morphological analyses of injured spinal cords have provided evidence of secondary tissue damage. Physiological studies indicate that many of the surviving axons are dysfunctional.

Histological Appearance of the Injury Site

Injured spinal cords show a remarkably consistent histological appearance regardless of injury. Shortly after injury, the spinal cords invariably show petechiae that progress over several hours to central hemorrhagic necrosis. After several weeks, the injury site typically develops an empty central cavity. Surviving axons are situated on the rim of this cavity, most densely concentrated 200 to 300 μm below the pial surface (1,2).

Normal cat spinal cords contain about 50,000 axons situated within 0.1 mm of the pial surface. The density of the axons increases slightly with depth. The total number of countable myelinated axons is approximately 500,000. Contusion injury of the spinal cord by dropping a 20-g weight from a height of 20 cm generally causes paralysis in 80% to 90% of cats. This insult dramatically reduces the number of axons, so that only about 15,000 axons remain within 0.1 mm of the pial surface (3). At greater distances from the pial surface, more dramatic losses of axons are apparent (Figure 1A). The gross histological appearance of the spinal cord often does not predict functional recovery (4).

Dysfunctional Axons

Axons surviving the contusion injury typically have reduced myelin thickness. This can be quantified by measuring the myelin index, defined as the ratio of the axon diameter to total fiber diameter (including myelin). The average myelin index in normal spinal cords is usually 0.5 to 0.6. Injury causes the ratio to approach 1.0 (Figure 1B). Demyelination plays a major role in axonal dysfunction in spinal cord injury. Spinal axons that have survived injury often cannot conduct impulses reliably (1).

To determine if the number of surviving spinal axons crossing lesion sites predicts function, we compared total axon counts with recovery of walking,

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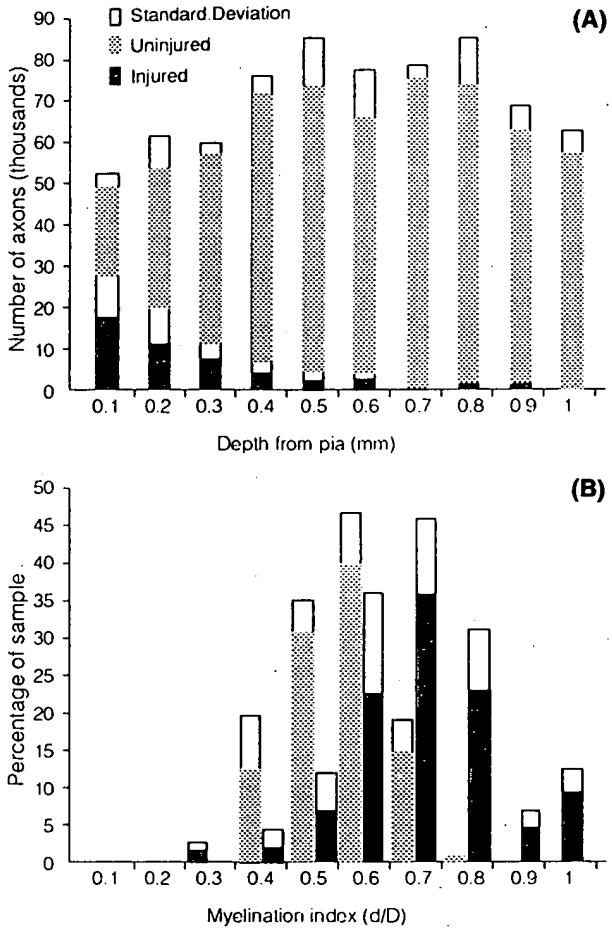


Figure 1. Number of axons (A) and axonal mylenation index (B) in cats with and without spinal cord injury. Reprinted with permission from Blight and Young (3).

free-fall responses representing vestibulospinal function, and somatosensory evoked potentials (Figure 2) (5). The data summarize several dozen studies conducted over a 10-year period. Injury dramatically reduced the number of countable axons from 500,000. In animals that were paralyzed from the injury, the mean axon count was 20,000, compared with 60,000 in those that recovered walking. This difference of about 40,000 axons is less than 10% of the normal population.

A substantial proportion of the surviving axons are likely to be demyelinated after spinal cord injury. Remyelination of injured axons improves conduction in the surviving axons (3). Therefore, the proportion of functioning axons needed to support motor recovery should be substantially less than 10%. Any treatment that can salvage or prevent demyelination of even a small percentage of spinal axons may dramatically improve functional recovery.

PHARMACOLOGICAL TREATMENT OF SECONDARY INJURY

Preclinical Studies

Preclinical studies play a key role in the success of clinical trials. The first National Acute Spinal Cord Injury Study (NASCIS 1) is a good illustration of the importance of systematic preclinical studies. In 1979 NASCIS 1 began to compare the effects of high- and low-dose methylprednisolone, a synthetic glucocorticoid drug, on human spinal cord injury. High-dose methylprednisolone consisted of an intravenous bolus of 1,000 mg followed by 1,000 mg/day for 10 days. Low-dose methylprednisolone was a 100 mg bolus followed by 100 mg/day for 10 days. The 330 patients enrolled in the study were all treated within 48 hours of injury. Evaluations performed at 6 months and 1 year postinjury did not show a significant difference in motor or sensory outcome between the two treatment groups (6,7).

The NASCIS 1 protocol was based largely on early laboratory studies reporting that methylprednisolone improved recovery in spinal cord-injured animals. Few of these studies did dose-response testing. None of the studies started methylprednisolone 24 to 48 hours postinjury, and none continued therapy for 10 days. Thus, the regimen tested by NASCIS had not been assessed in any model of spinal cord injury. New evidence (8–12), obtained while NASCIS 1 was being carried out, suggested that too little

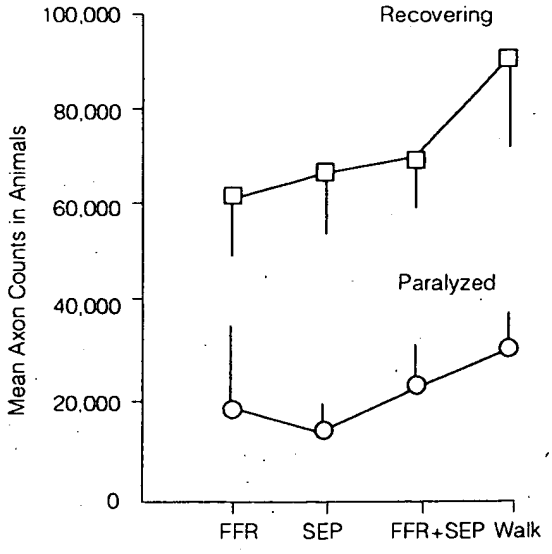


Figure 2. Mean axon counts in animals with and without functional recovery following spinal cord injury. Reprinted with permission from Young and Mallin (5).

methylprednisolone was given too late. Animal studies suggested that the optimal dose was 30 mg/kg, twice the "high dose" of methylprednisolone used in NASCIS 1.

Clinical Studies

The second National Acute Spinal Cord Injury Study (NASCIS 2) started in 1985 (13–17) and randomized 487 patients to treatment with methylprednisolone, naloxone, or placebo. The methylprednisolone protocol (30 mg/kg followed by 5.4 mg/kg/h for 23 hours) was based on studies showing that 30 mg/kg optimally inhibited lipid peroxidation. The naloxone protocol (5.4 mg/kg followed by 3.0 mg/kg/h for 24 hours) was based on laboratory work showing that 2 mg/kg/h (18) or a 10 mg/kg bolus dose (19, 20) improved recovery in spinal cord-injured animals. Patients with a spinal cord injury will tolerate 5.4 mg/kg well (21). The maintenance doses were based on the plasma half-life of the drug in humans. The treatment duration of 24 hours was chosen to avoid complications of prolonged glucocorticoid therapy. Motor and sensory functions were assessed at 6 weeks, 6 months, and 1 year postinjury.

Patients who received methylprednisolone within 8 hours of injury consistently had better sensory and motor function than did those who received placebo. Differences in motor improvement between methylprednisolone- and placebo-treated patients were statistically significant at all three follow-up periods. At 6 weeks and 6 months postinjury, improvement in touch sensation was significantly greater in patients receiving early methylprednisolone than in those receiving placebo. Likewise, pinprick sensory recovery was significantly better in methylprednisolone-treated patients than in placebo-treated patients. Patients who received naloxone or those who received methylprednisolone more than 8 hours postinjury did not differ significantly from the placebo-treated group.

The effect of methylprednisolone on functional recovery was examined separately in "plegic" patients who had no residual motor or sensory function below the lesion level at the time of admission and "paretic" patients who had some motor or sensory function below their injury level. Although paretic patients treated early with methylprednisolone recovered more function than did plegic patients, both groups benefitted from methylprednisolone therapy. Recovery scores of the naloxone group consistently fell between the methylprednisolone and placebo group. At each assessment interval, the methylprednisolone group showed the greatest recovery of sensory and motor function (Figure 3) (13). At the 1-year evaluation, plegic patients had recovered 21% of what they had lost, while paretic patients recovered 75%. Placebo-treated plegic and paretic patients, respectively, recovered 59% and 8% of what they had lost on admission.

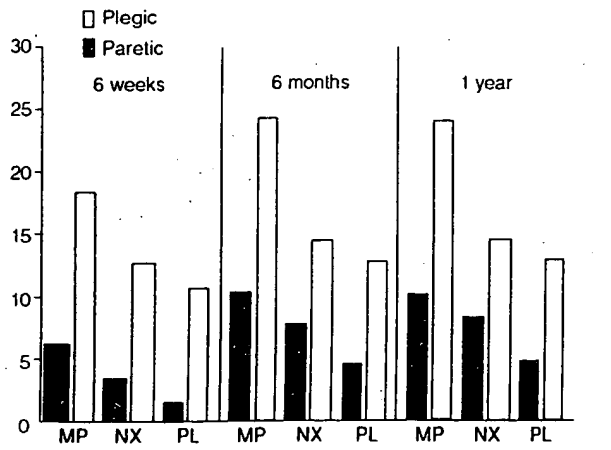


Figure 3. Changes in motor function scores 6 weeks, 6 months, and 1 year after spinal cord injury in plegic and paretic patients treated with methylprednisolone, naloxone, or placebo. Treatment was given within 8 hours. Reprinted with permission from Bracken and colleagues (7).

IMPLICATIONS FOR CLINICAL AND PRECLINICAL RESEARCH

NASCIS 2 identified several critical variables that influence treatment effect and have important implications for spinal cord injury research.

Dose Response

The methylprednisolone dose used in NASCIS 2 was derived from laboratory studies showing that 30 mg/kg of methylprednisolone best inhibited lipid peroxidation (8,9,11,22,23), increased posttraumatic blood flow (24), and improved locomotory recovery (20). Higher doses of 60 to 90 mg/kg, paradoxically, may increase spinal cord damage. Other drugs show similar biphasic dose-response curves, characterized by a threshold, a narrow peak, and reversal to deleterious effects at higher doses (25,26).

The dose-response of methylprednisolone underscores the need for detailed preclinical studies to identify the optimal therapeutic dose. A major disadvantage is a narrow dose-response curve with potentially deleterious effects at higher doses. Drugs with wider

dose-response curves and no reversal of effect at higher doses would be safer and easier to administer.

Treatment Initiation Time

In NASCIS 2, the patients were all randomized and treated within 12 hours. The median treatment time was about 8 hours. About half of the patients were treated within 8 hours postinjury and the other half between 8 and 12 hours postinjury. Therefore, for data analysis purposes, the patients were stratified into two treatment groups: early (≤ 8 hours) or late (>8 hours).

The optimal treatment initiation time requires further investigation. Future studies should focus not only on the benefits of early treatment but also on possible risks of late initiation. At the 1-year follow-up period, patients who received methylprednisolone late had worse neurological recovery than placebo-treated patients. NASCIS 3 is randomizing patients for treatment within 6 hours of injury and will be comparing earlier treatment periods.

The optimum treatment time may differ, depending on the drug and its mechanism of action. In a recently published experimental study, the 21-aminosteroid tirilazad mesylate, given within 4 hours postinjury, improved functional recovery in cats (27). No beneficial effect was observed, however, when tirilazad mesylate was administered 8 hours after injury. Conversely, the monosialic ganglioside, GM-1 ganglioside, was shown to improve functional recovery even when treatment was delayed for 48 to 72 hours after spinal cord injury (28,29).

Treatment Duration

Secondary tissue damage is believed to continue for days after spinal cord injury. For this reason, NASCIS 1 randomized patients as late as 48 hours postinjury and treated them for 10 days. Subsequent experiences, particularly NASCIS 2, suggest that rapid treatment initiation may be more important for favorable outcome than treatment duration. The delay in treatment initiation in NASCIS 1 may well have contributed to the negative findings. The long treatment duration also contributed to increased side effects of glucocorticoid administration.

The optimal duration of neuroprotective therapy is an issue that has been largely neglected in preclinical studies conducted to date. In animal experiments, treatments are usually given for 3 to 6 hours postinjury. NASCIS 2 used a 24-hour treatment duration for safety reasons. NASCIS 1 showed that a 10-day course of methylprednisolone increases the infection rate. Since the NASCIS 2 dose was much higher and the drug is likely to linger for some time, the group decided to limit the treatment period to 24 hours.

Spinal cord edema and demyelination do not reach a peak until several days after injury. The delay in these reactions to injury suggests that secondary tissue damage continues to progress for more than 24 hours. Consequently, administration of methylprednisolone for a longer period may provide greater benefit. NASCIS 3 will compare 24- and 48-hour durations of methylprednisolone infusion with 48 hours of tirilazad mesylate infusion. All the patients will receive the same initial 30 mg/kg dose of methylprednisolone within 6 hours after spinal cord injury.

Injury Severity

Neuronal regeneration has long been regarded as the only approach to restoring neurological function after severe spinal cord injury. However, loss of function does not necessarily mean loss of axons. Likewise, recovery does not necessarily imply regeneration. For example, one study (30) described patients with spinal cord arteriovenous malformations, which leave some patients paralyzed for several years with no somatosensory evoked potentials. Embolization of the malformation usually results in rapid and long-lasting improvement. Such rapid recoveries are unlikely to be due to regeneration.

NASCIS 2 confirmed the potential of severely spinal cord-injured patients to recover substantial function. This finding challenges a long-held clinical dogma that such patients cannot recover. Plegic patients given early methylprednisolone recovered significantly more than placebo-treated patients. At 6 weeks after spinal cord injury, motor scores in plegic patients rose by 6.2 points, compared with 1.3 points in placebo-treated patients. Pinprick and touch-sensation changes showed a similar pattern. Methylprednisolone continued to be better than placebo at 6-month and 1-year follow-up.

The improvement of both plegic and paretic patients observed in NASCIS 2 underscores the need to include injury severity in the trial design and data analysis. Likewise, preclinical studies must examine treatments over a range of injury severity. Pharmacological therapies that are effective in mild spinal cord injury may have less effect on severe injuries and may even cause deleterious effects. Alternatively, some treatments may be beneficial only in severe injuries. Finally, blood flow changes and vascular damage associated with severe injuries may alter drug penetration into the injury site.

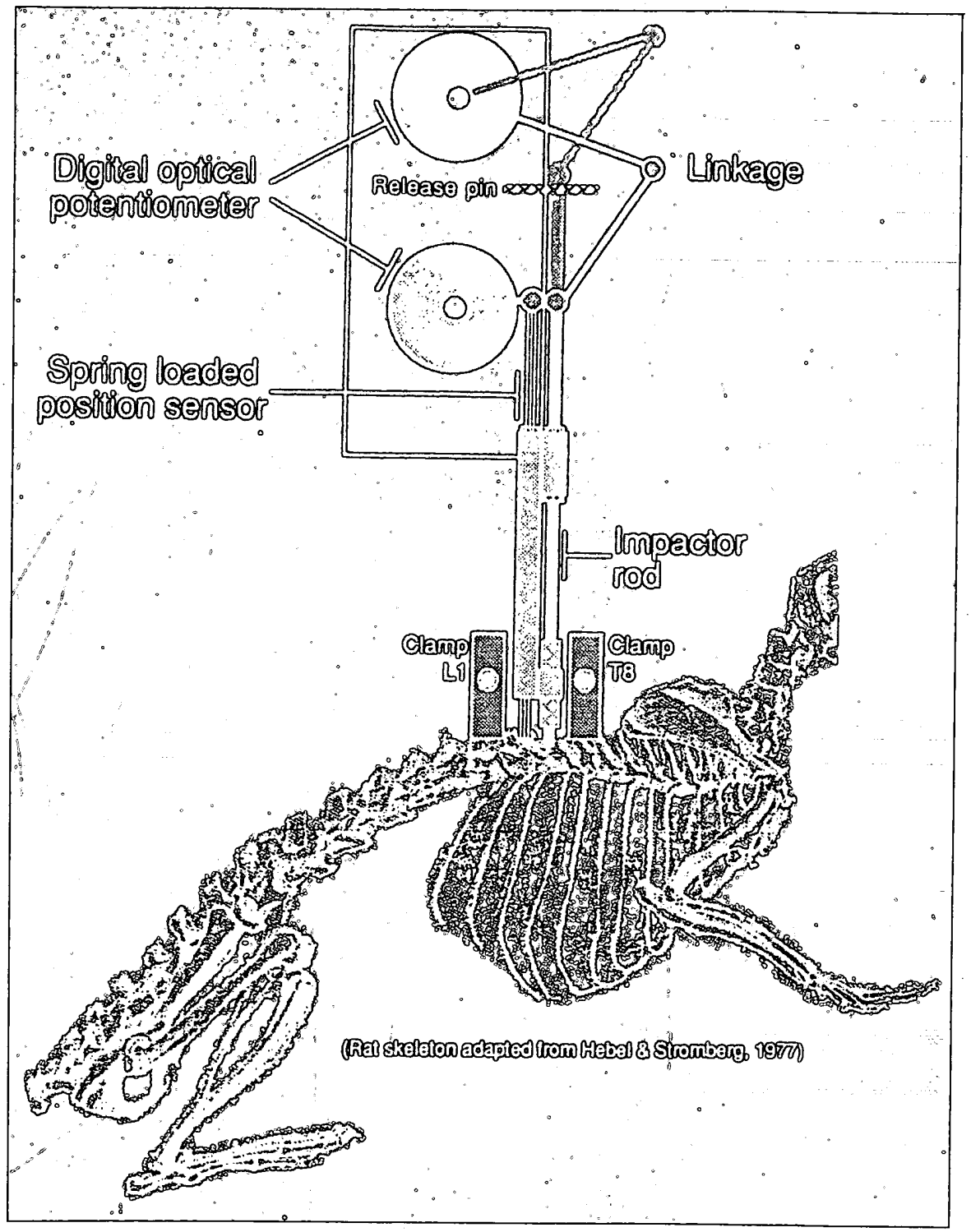


Figure 4. Schematic diagram showing components of rat spinal contusion device.

Combination Therapy

Because methylprednisolone significantly improves functional recovery, placebo trials are no longer feasible or recommended. A proposed therapy must now be shown to be superior to methylprednisolone before it can be tested in a clinical trial. However, both patients and clinical investigators may hesitate to substitute a new treatment for a proven one. As a result, combined treatment with methylprednisolone may be considered seriously in the trial design.

In addition to concomitant administration, se-

quential therapy must be considered. Most of the clinical trials that have been planned and initiated after NASCIS 2 have included at least one treatment arm where an initial bolus dose of 30 mg/kg methylprednisolone is given, followed by a second drug or dose. For example, NASCIS 3 will be assessing an initial bolus of methylprednisolone followed by 48 hours of tirilazad mesylate. Another multicenter study will be assessing a 24-hour course of methylprednisolone followed by 4 to 6 weeks of GM-1 ganglioside, compared with methylprednisolone alone.

Combination therapy cannot be avoided in critically ill patients. Almost all spinal cord-injured patients will be receiving multiple drugs with vasoactive and other effects that may interact with methylprednisolone. Preclinical investigation of putative neuroprotective therapies must take this factor into account and should incorporate such interactions into the treatment protocols. For example, many spinal cord-injured patients receive dopamine to maintain blood pressure, and the interactions of dopamine and methylprednisolone should be assessed.

FUTURE OF SPINAL CORD INJURY RESEARCH

NASCIS 2 has greatly complicated spinal cord injury research. A new treatment must be shown to be equal or superior to methylprednisolone to become eligible for clinical trial. Because differences between any treatment and methylprednisolone will be smaller than differences between methylprednisolone and placebo controls, clinical trials must assess larger populations of patients.

Preclinical Studies

The NASCIS results have placed an inordinate burden on preclinical studies of new spinal cord injury therapies. For example, assessment of three treatment doses at three initiation times and three durations, over three injury severities—with appropriate methylprednisolone, vehicle, and sham controls, as well as combination therapy—adds up to a minimum of 270 experimental groups. The best spinal cord injury models and outcome measures available today can detect a methylprednisolone effect compared with placebo with 10 to 20 animals per group. Systemic investigation of a single treatment thus will require thousands of experiments.

Individual laboratories do not have the resources

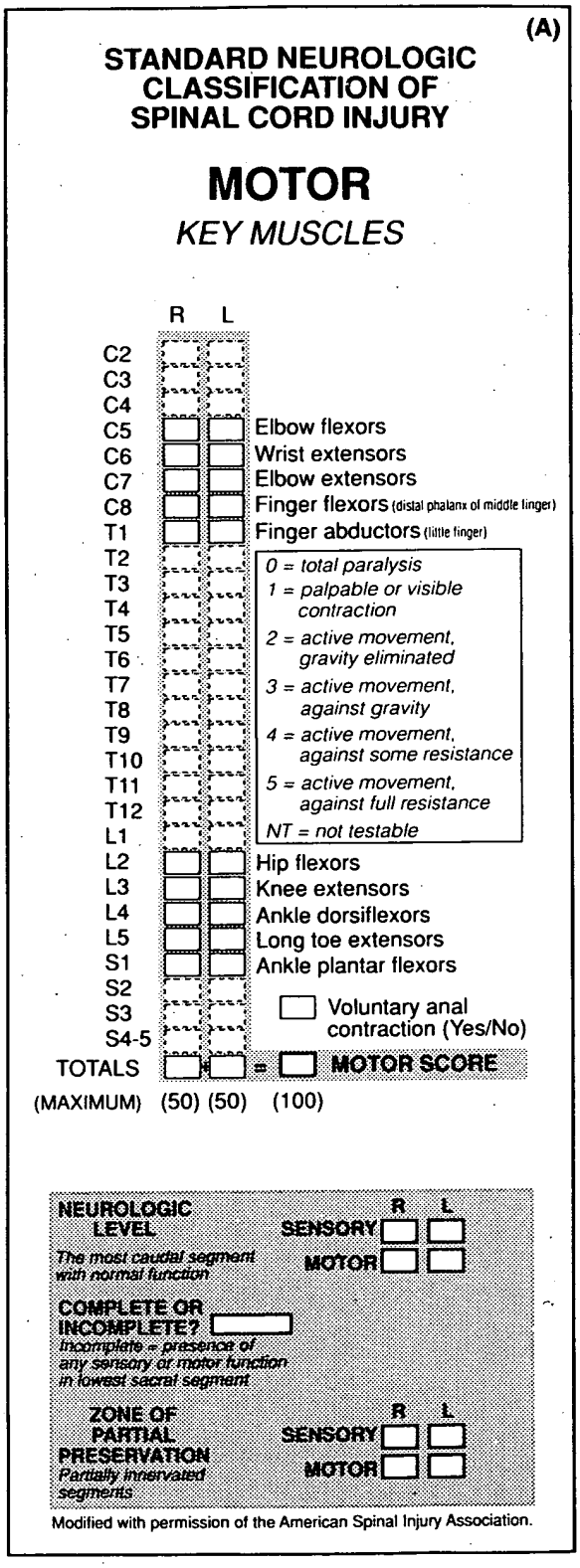


Figure 5. The 1992 Standard Neurological Classification of Spinal Cord Injury. (A) Motor (key muscles). (B) Sensory (key sensory points). Modified with permission from the American Spinal Injury Association (33). Copies of the standard can be obtained by contacting: American Spinal Injury Association, c/o Lesley M. Hudson, MA, 2020 Peachtree Road, NW, Atlanta, GA 30309.

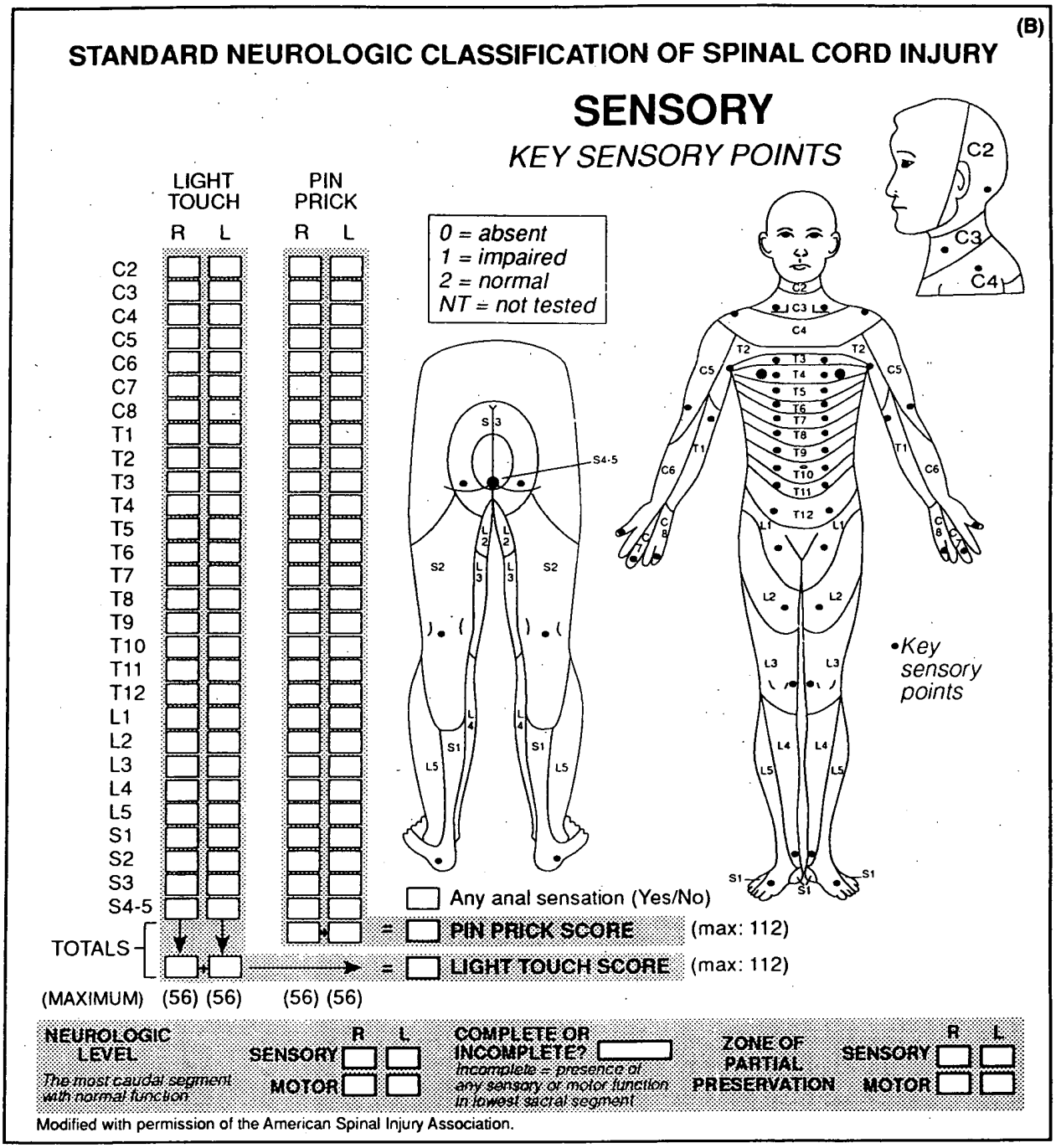
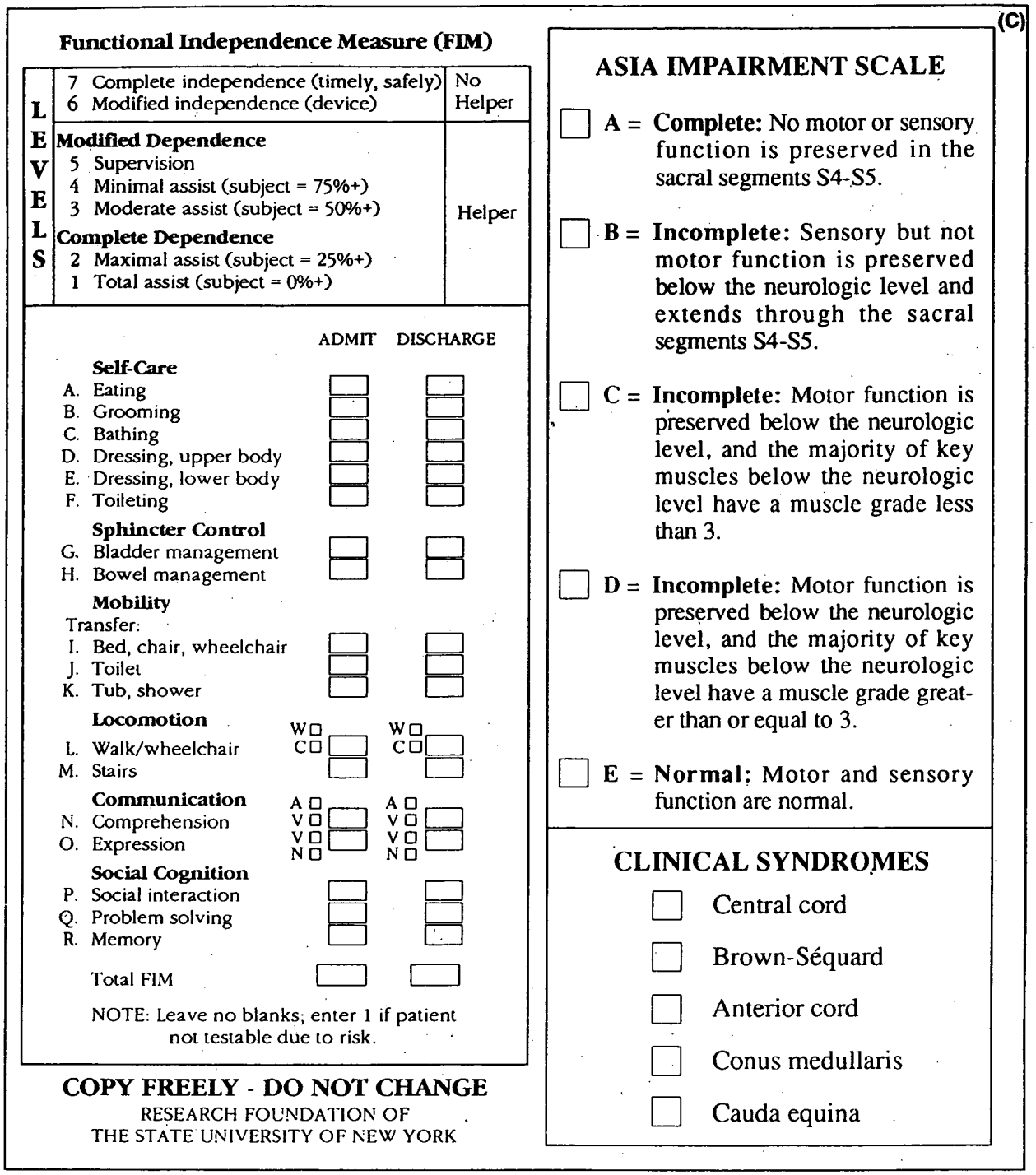


Figure 5. Continued.

and capabilities to carry out investigations of this magnitude. Multicenter preclinical spinal cord injury studies are necessary to spread the research burden across institutions. Treatments must be screened to identify the best treatment protocols for testing with time-consuming behavioral and histological outcome measures. Better, standardized injury models and outcome measures are essential to the success of such an undertaking.

New York University Animal Spinal Cord Injury Model

To answer this challenge, the New York University Neurosurgery Laboratory has developed an efficient and reproducible rat spinal cord injury model. The injury is caused by dropping a rod onto thoracic spinal cord (Figure 4) exposed by laminectomy. Digital optical potentiometers are used to monitor rod and



Functional Independence Measure (FIM)

LEVELS

	7 Complete independence (timely, safely)	No Helper
	6 Modified independence (device)	
	Modified Dependence	Helper
	5 Supervision	
	4 Minimal assist (subject = 75%+)	
	3 Moderate assist (subject = 50%+)	
	Complete Dependence	
	2 Maximal assist (subject = 25%+)	
	1 Total assist (subject = 0%+)	

	ADMIT	DISCHARGE
Self-Care		
A. Eating	☐	☐
B. Grooming	☐	☐
C. Bathing	☐	☐
D. Dressing, upper body	☐	☐
E. Dressing, lower body	☐	☐
F. Toileting	☐	☐
Sphincter Control		
G. Bladder management	☐	☐
H. Bowel management	☐	☐
Mobility		
Transfer:		
I. Bed, chair, wheelchair	☐	☐
J. Toilet	☐	☐
K. Tub, shower	☐	☐
Locomotion		
L. Walk/wheelchair	W☐ C☐	W☐ C☐
M. Stairs	☐	☐
Communication		
N. Comprehension	A☐ V☐	A☐ V☐
O. Expression	V☐ N☐	V☐ N☐
Social Cognition		
P. Social interaction	☐	☐
Q. Problem solving	☐	☐
R. Memory	☐	☐
Total FIM	☐	☐

NOTE: Leave no blanks; enter 1 if patient not testable due to risk.

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(C)

ASIA IMPAIRMENT SCALE

☐ A = **Complete:** No motor or sensory function is preserved in the sacral segments S4-S5.

☐ B = **Incomplete:** Sensory but not motor function is preserved below the neurologic level and extends through the sacral segments S4-S5.

☐ C = **Incomplete:** Motor function is preserved below the neurologic level, and the majority of key muscles below the neurologic level have a muscle grade less than 3.

☐ D = **Incomplete:** Motor function is preserved below the neurologic level, and the majority of key muscles below the neurologic level have a muscle grade greater than or equal to 3.

☐ E = **Normal:** Motor and sensory function are normal.

CLINICAL SYNDROMES

☐ Central cord

☐ Brown-Séquard

☐ Anterior cord

☐ Conus medullaris

☐ Cauda equina

Figure 5. Continued.

vertebral movements during the impact. Subtracting vertebral movements from rod movements allows precise measurements of the extent and time course of spinal cord compression. By using analysis of covariance (ANCOVA) with spinal cord compression rate as the covariate, effects of injury variability can be minimized.

Treatment evaluation is carried out in two stages. The initial stage uses spinal cord sodium and potassium changes to quantify tissue damage (31). The

ionic shifts reflect lesion volumes at the impact site. Tissue ionic shifts can be accurately and rapidly measured at 24 hours postinjury. This approach allows rapid screening of many treatment protocols to identify the best dose, timing, and duration for three injury severities. In the second stage, the best treatment protocols will be tested with time-consuming behavioral and quantitative histological analyses of chronically spinal cord-injured animals. This will be carried out by a consortium of spinal cord injury centers using the same injury model, treatment protocols, and outcome measures.

This approach toward evaluating new spinal cord injury therapies will increase the efficiency of preclinical studies. Screening therapies to identify optimal treatment parameters reduces the number of experiments by an order of magnitude. Testing the animals through a multicenter consortium spreads the burden of research across several institutions.

An International Classification Standard for Spinal Cord Injury

NASCIS 2 has likewise complicated spinal cord injury clinical trials. Because an effective treatment has been identified, placebo-controlled trials can no longer be justified. New treatments must be compared against methylprednisolone. Treatment differences will be small and more difficult to detect. Physicians and patients also are reluctant to eschew a treatment of proven effectiveness. For this reason, multicenter spinal cord injury clinical trials now under way include methylprednisolone in all treatment arms.

A major obstacle to clinical trials has been the hodgepodge of clinical classifications and outcome measures used by clinicians to assess spinal cord injury. The American Spinal Injury Association (ASIA) has long advocated a scoring approach that is widely used by orthopedic and rehabilitation groups (32). Neurosurgeons have favored the NASCIS approach. The major clinical data bases were incompatible, and comparison of results from different centers was difficult.

Recently, the major organizations in the field worked together to establish the first International Spinal Cord Injury Classification Standard (33). This standard unified the ASIA and NASCIS scoring approaches, defined spinal cord injury levels and syndromes, specified an objective impairment scale, and recommended a functional assessment instrument. All major spinal cord injury clinical trials now under way in the United States and Europe have adopted the standard (Figure 5). The standard allows clinical centers to share data and will greatly facilitate clinical trials of treatment of spinal cord injury.

Directions for Future Research

The success of methylprednisolone in improving neurological function following spinal cord injury is expected to lead to evaluation of many compounds. Multicenter clinical trials are already under way with two promising agents—tirilazad mesylate and GM-1 ganglioside. In addition, a number of agents such as antioxidants, opiate receptor antagonists, neurotransmitter receptor blockers, anti-inflammatory agents, and enzyme blockers have been identified that have the potential for ameliorating the secondary damage associated with spinal cord injury. Systematic preclinical evaluation of these compounds, followed by well-designed clinical trials, will lead to safer and more effective therapies for spinal cord injury.

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Effect of yohimbine on nerve growth factor mRNA and protein levels in rat hippocampus

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Key words: Yohimbine; Nerve growth factor mRNA; Nerve growth factor protein; Noradrenergic stimulation

Activation of noradrenergic receptors has been shown to increase expression of nerve growth factor (NGF) gene in brain cells in vitro. The present studies were undertaken to determine if this stimulation was effective in vivo as well. Rats were administered the norepinephrine-releasing drug, yohimbine (YOH), and had their hippocampi assayed for NGF mRNA and protein at various times after the injection. It was found that yohimbine caused a 3-fold increase of NGF mRNA levels at 24 h. Protein levels, however, were unaltered at this time. Thus norepinephrine release in vivo appears to be sufficient for increasing mRNA level but not for translation to protein.

The noradrenergic neuronal system of the brain has been implicated in a number of long-term processes in the CNS. These include learning [7,12], neuronal plasticity [14], behavioral sparing [10], recovery after lesion [2], neuronal differentiation [4,11] and adaptation to chronic stress [16]. How the noradrenergic system accomplishes these actions is not fully understood. It has been found, however, that this system activates a number of immediate early genes in the brain including c-*fos*, *nur*77, *zif*-268, *tis*-7 and *tis*-21 [1,6]. Among the target genes for one of these immediate early genes (c-*fos*) is nerve growth factor (NGF) which could mediate certain of the above long-term processes [9]. It has been found that levels of NGF mRNA and protein in brain cell cultures can be increased by stimulation of beta adrenoceptors [5,13]. We were therefore interested in determining whether the NGF gene could be activated by noradrenergic stimulation in vivo, and if so, whether this would be accompanied by elevation of brain NGF protein levels. To accomplish this we administered rats yohimbine (YOH), an alpha-2 adrenergic antagonist which causes activation of the locus coeruleus and the release of NE in the brain [17], and assayed for NGF mRNA and protein in the hippocampus.

Rats were housed 2 per cage and maintained on a 12 h light/dark cycle (lights on 07.00 h). The animals were habituated to the laboratory for 2–3 days. YOH was administered i.p. at 5 mg/kg. The animals were sacrificed by decapitation between 1 and 48 h after the injection. Hippocampal RNA was prepared according to a previously published method [3] and quantified spectrophotometrically. Purity was checked on a formaldehyde-denatured agarose gel. A commercial kit (Ambion) was used for the ribonuclease protection assay (RPA). The [^{32}P]riboprobe was directed against a 300 bp fragment of the rat NGF gene in the 5′ non-coding and coding regions. Northern analysis indicated that the probe bound with a single discrete band at the expected position of NGF mRNA. The hybridization and treatment with ribonuclease were performed as specified by the manufacturer. The protected fragment was separated on a polyacrylamide gel and visualized by autoradiography. The autoradiograms were scanned densitometrically using exposures that were within the linear range. For purposes of control, beta actin mRNA was assayed by the RPA as well. The probe for beta actin was made from the template supplied with the RPA kit. NGF protein was assayed in independent groups of animals by an ELISA as previously described [15] with the modification that the capture antibody was polyclonal (Becton-Dickinson) and used at a concentration of 0.5 μg/ml. The assay was linear over the range 1–100 pg and employed an average of 5 mg of tissue.

The effect of YOH on hippocampal NGF mRNA levels is shown in Fig. 1. ANOVA revealed a significant effect of YOH ($F_{1,30} = 11.09$, $P < 0.005$). Post-hoc (Newman–Keuls) comparisons revealed that the levels in the YOH treated rats were greater than those in the controls at the 24 h time point ($P < 0.005$). Beta-actin mRNA,

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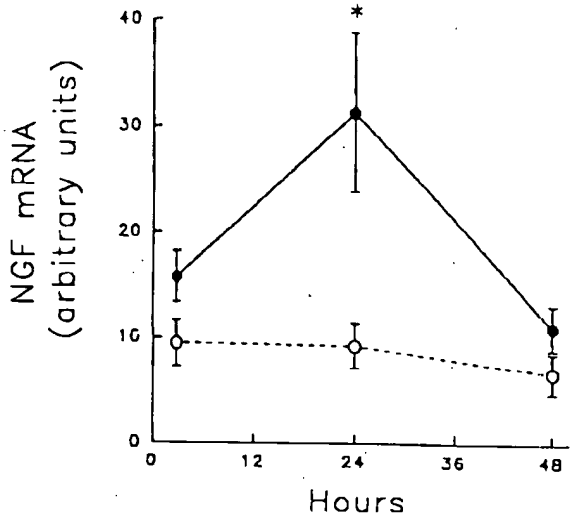

Fig. 1. Effect of YOH, 5 mg/kg i.p., on NGF mRNA levels in hippocampus. Controls (dashed line), YOH-treated (solid line). Values are means ± S.E.M. of 6 rats. $^{*}P < 0.005$ versus control group.

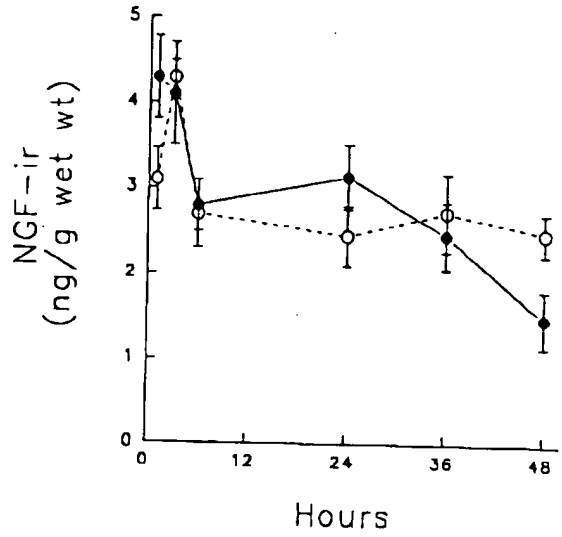

Fig. 2. Effect of YOH on NGF protein in hippocampus. Values are means ± S.E.M. of 3–6 rats. Controls (dashed), YOH (solid).

assayed in separate groups of animals given either saline or YOH 24 h previously, was unaffected (data not shown).

The effect of YOH on hippocampal NGF protein is shown in Fig. 2. There was no significant effect of YOH on these values.

The present results indicate that YOH administration increases the levels of NGF mRNA in the rat hippocampus. A significant increase of 3-fold was found at 24 h post-injection. These findings confirm the in vitro findings cited above showing that catecholamine stimulation can increase NGF mRNA in the brain in vivo. These data do not reveal the mechanism of the increase which could be an effect on gene activation or on the stability or turnover of NGF mRNA.

A discrepancy was found, however, between the levels of mRNA and protein. NGF protein was not increased above control levels in the YOH-treated rats at these intervals. A similar discrepancy has been reported in the rat dorsal hippocampus for kainic acid which causes an increase in NGF mRNA but a decrease in NGF protein [8,18]. The reason for this discrepancy is not presently clear. It may, however, indicate that catecholamine activation represents a necessary but insufficient stimulus for NGF protein synthesis. Other factors may be required to cause translation of the increased mRNA signal into protein.

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NEURO-SCIENCE LETTERS

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Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord

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Keywords: blood–brain barrier, macrophage recruitment, neutrophil recruitment

Abstract

Lesion-induced inflammatory responses in both brain and spinal cord have recently become a topic of active investigation. Using C57BL/6J mice, we compared the tissue reaction in these two central nervous system (CNS) compartments with mechanical lesions of similar size involving both grey and white matter. This evaluation included the quantitative assessment of neutrophils, lymphocytes and activated macrophages/microglia, as well as astrocyte activation, upregulation of vascular cell adhesion molecules (ICAM-1, VCAM-1, PECAM) and the extent of blood–brain barrier (BBB) breakdown. Time points analysed post-lesioning included 1, 2, 4 and 7 days (as well as 10 and 14 days for the BBB). We found clear evidence that the acute inflammatory response to traumatic injury is significantly greater in the spinal cord than in the cerebral cortex. The numbers of both neutrophils and macrophages recruited to the lesion site were significantly higher in the spinal cord than in the brain, and the recruitment of these cells into the surrounding parenchyma was also more widespread in the cord. The area of BBB breakdown was substantially larger in the spinal cord and vascular damage persisted for a longer period. In the brain, as in spinal cord, the area to which neutrophils were recruited correlated well with the area of BBB breakdown. It will be of interest to determine the extent to which the infiltration of inflammatory cells contributes, either directly or indirectly, to the vascular permeability and secondary tissue damage or, conversely, to local tissue repair in the brain and the spinal cord.

Introduction

Traumatic central nervous system (CNS) injury initiates a complex cascade of events, prominent among which are immediate local ischaemia and both immediate and delayed cell death (Tator, 1991; Tator *et al.*, 1995; Schwab & Bartholdi, 1996). Until recently, little attention has been paid to the acute inflammatory response which follows CNS trauma. Although the inflammatory response seen in the CNS following different types of external challenge has similarities to the response seen in non-neuronal tissues, there are clear differences (Perry *et al.*, 1995). In general, an equivalent stimulus leads to a much weaker response in the CNS parenchyma. A good example is the challenge with lipopolysaccharide (LPS), a potent inflammatory agent, which in nanogram amounts evokes a robust reaction in the periphery including massive leukocyte recruitment. When injected into the murine brain, however, LPS causes only a minor vascular response, minimal recruitment of neutrophils and delayed monocyte invasion (Andersson *et al.*, 1992). The reason for this difference in response pattern is still unclear.

When considering the possible factors contributing to CNS-specific responses to trauma, several unique aspects of the CNS tissue become apparent. First of all, under normal conditions, the blood–brain barrier (BBB) largely excludes serum proteins from the extracellular milieu. In addition, microglia, the resident macrophages of the brain, normally express a quiescent phenotype (Lawson *et al.*, 1993), and there is an absence of typical extracellular matrix (Rutka *et al.*, 1988). On the other hand, many molecules necessary for the onset of a successful inflammatory cascade are present in the CNS parenchyma; cell adhesion molecules are either constitutively expressed on the vascular endothelium or rapidly upregulated upon challenge (Engelhardt *et al.*, 1994; Bell & Perry, 1995), and both cytokines and chemokines are also expressed in brain tissue, either constitutively or following trauma (Pousset, 1994; Glabinski *et al.*, 1996; Bartholdi & Schwab, 1997; Ransohoff & Tani, 1998; for review, see Toulmond *et al.*, 1996).

Most studies of lesion-related inflammation in the CNS have been carried out in the brain. Interestingly, recent studies on the spinal cord suggest that the responses of these two CNS compartments differ with a stronger inflammatory reaction being evoked in the spinal cord (Dusart & Schwab, 1994; Holmin *et al.*, 1995; Carlson *et al.*, 1998). A direct comparison between studies is difficult, however, given the many methodological differences, including strains and species of animal used and lesioning techniques applied, as well as the parameters chosen to evaluate the inflammatory response.

The present study was designed to provide a systematic comparison of the inflammatory sequelae of brain and spinal cord lesions. Using a single animal strain, the lesion technique was standardized so as to involve both grey and white matter. The same parameters were used for all histological evaluations, and the time points of investigation were set at 1, 2, 4 and 7 days after the lesion. Leukocyte recruitment was broken down into cell type (neutrophils,

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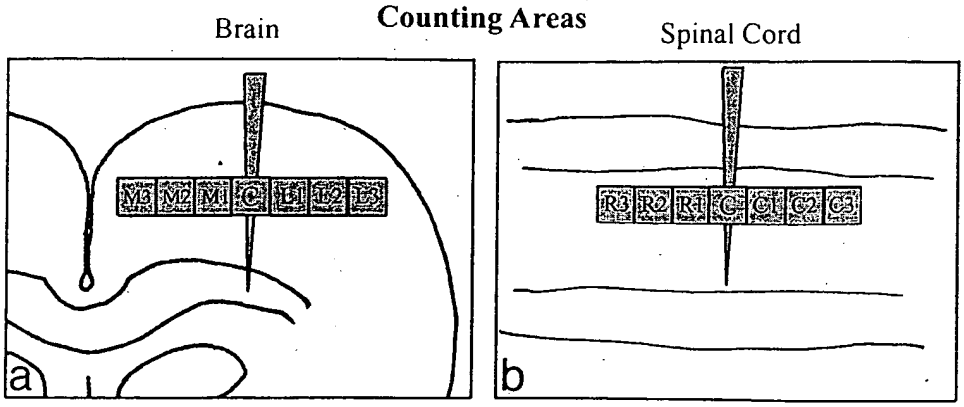

FIG. 1. Schematic drawing of counting areas in the brain (a) and spinal cord (b) in relation to the lesion (arrowhead). One square is 0.02 mm^2.

lymphocytes and macrophages). Astrocyte and endothelial activities were examined, as was BBB status.

Materials and methods

Animals

Mice of the C57BL/6J strain, 2–3 months old, were obtained from a specific pathogen-free (SPF) breeding colony (Olac, UK). Male and female animals were used in this experiment, and maintained on a standard regime with food and water *ad libitum*. The animals were anaesthetized with i.p. hypnorm (Janssen Laboratories; 0.25 mg fluanisone per 20 g body weight, total volume 250 µL) and hypnovel (Roche; 0.25 mg midazolam per 20 g body weight, total volume 250 µL), and subjected either to spinal cord lesions or to brain lesions. After surgery the animals were kept on a thermostatically regulated heating plate until completely awake. All procedures were carried out in accordance with the requirements of the UK Animals (Scientific Procedures) Acts (1986).

Brain lesions

After the skin overlying the skull was incised, a craniotomy was created which was centred on the coronal suture over the right hemisphere. Iridectomy scissors were then used to make a parasagittal incision in the frontal cortex, 2.5 mm in length, 1 mm lateral to the midline and 2–3 mm deep, cutting through the dura and both grey and white matter of the brain parenchyma, but sparing prominent meningeal vessels. Gelfoam (Upjohn Company Kalamazoo, MI, USA) was used when necessary to achieve haemostasis. At the completion of surgery the skin was reapposed with wound clips.

Spinal cord lesions

The skin overlying the vertebral column was incised, the muscles detached from the vertebrae and a partial laminectomy performed at T8. Special care was taken to minimize injury to vessels of the lateral plexus. After opening the dura, the spinal cord was lesioned using fine iridectomy scissors so as to produce a bilateral lesion of the dorsal funiculi, the corticospinal tracts and part of the dorsal horns. The resultant bleeding was usually minor and easily managed with Gelfoam. The muscles were reapposed with sutures and the skin closed with wound clips. In all cases recovery was uneventful, except for the typical transient paresis of the hindlimbs seen during the first few hours following surgery.

Tissue preparation

Unfixed tissue was obtained for lymphocyte immunohistochemistry at 1, 2, 4 and 7 days following lesioning. After an overdose of halothane (Rhone Merieux Ltd, Harlow, Essex, UK), the mice were decapitated and the spinal cords or brains dissected out, embedded in Tissue Tek OCT (Miles Laboratories, Elkhart, IN, USA) and snap frozen in isopentane at –40 °C. A minimum of three animals was studied for each time point.

Transcardiac perfusions were carried out with periodate-lysine paraformaldehyde containing 0.05% glutaraldehyde (PLP) (McLean & Nakane, 1974) for all tissue undergoing immunohistochemical staining for specific antigens. A total of 18 animals with brain lesions and 18 animals with spinal cord lesions were studied at 1, 2, 4 or 7 days after lesioning. Perfusion with Karnovsky's fixative (1% formaldehyde and 1.25% glutaraldehyde) was employed in other animals ($n = 52$), where the breakdown of the BBB was to be evaluated. In these animals, two additional time points, 10 and 14 days post-lesion, were added. After dissection, all brains and spinal cords were postfixed for several hours in the appropriate fixative and then immersed in 25% sucrose buffer, until sunk, for cryoprotection. The tissue was then embedded in Tissue Tek OCT and frozen in isopentane at –40 °C.

Series of coronal sections of brain and parasagittal sections of spinal cord were cut at 15 µm (30 µm for the BBB studies) on a cryostat and mounted on gelatinized slides. For immunohistochemical staining, an indirect three-step alkaline phosphatase (Schnell *et al.*, 1997) or avidin–biotin method was used (Lawson *et al.*, 1990).

Damage to the BBB was investigated by intravenous injection of 100 µL of 5% horseradish peroxidase (Sigma, peroxidase, Type II, in 0.9% saline) 30 min prior to perfusion (Claudio *et al.*, 1990; Hawkins *et al.*, 1990). Mounted sections were then processed using a modified Hanker Yates method (Perry & Linden, 1982). Using an ImageGrabbing System and Grafitek Image Analysis Program (Optilab/24 version 2.1) the area of HRP leakage was measured in three tissue sections from each brain or spinal cord, and the mean value of at least three animals per time point was calculated.

Immunohistochemistry

Primary antibodies used for labelling specific cell types.

Neutrophils

A polyclonal antiserum raised in rabbits (HB 199) against isolated rat neutrophils (Anthony *et al.*, 1997) was used at a dilution of 1 : 2000 for immunohistochemistry.

Activated microglia/macrophages

The polyclonal antibody F4/80, which detects a mononuclear phagocyte-specific cell surface antigen (McKnight *et al.*, 1996), and the monoclonal antibody FA/11 (CD68) (Rabinowitz & Gordon, 1991), which recognizes a membrane sialoglycoprotein widely

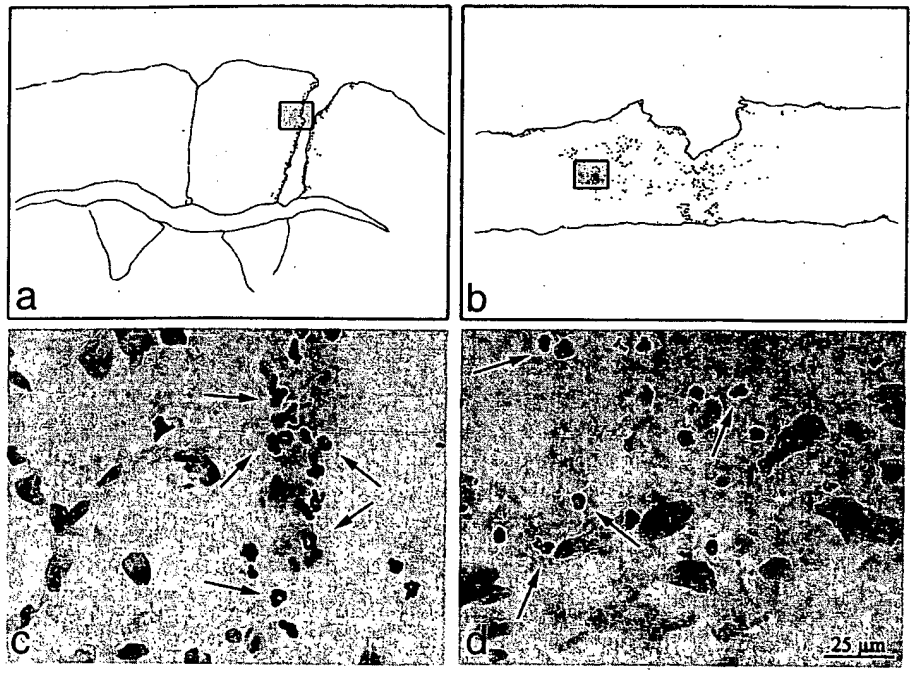

FIG. 2. Neutrophils in the brain and spinal cord at 1 day after lesion. (a and b) Superimposed camera lucida drawings of four consecutive sections (60 µm) of the brain (a) and spinal cord (b), showing the distribution of neutrophils in the vicinity of the lesion. Cells were identified by cresyl violet staining and/or with a polyclonal antiserum (HB-199) against rat neutrophils. (c and d) Cresyl violet-stained section corresponding to the boxed region in (a and b). In the brain (c), neutrophil infiltration is restricted to the rim of the lesion. In contrast, neutrophils in the spinal cord (d) infiltrate the parenchyma over a large area.

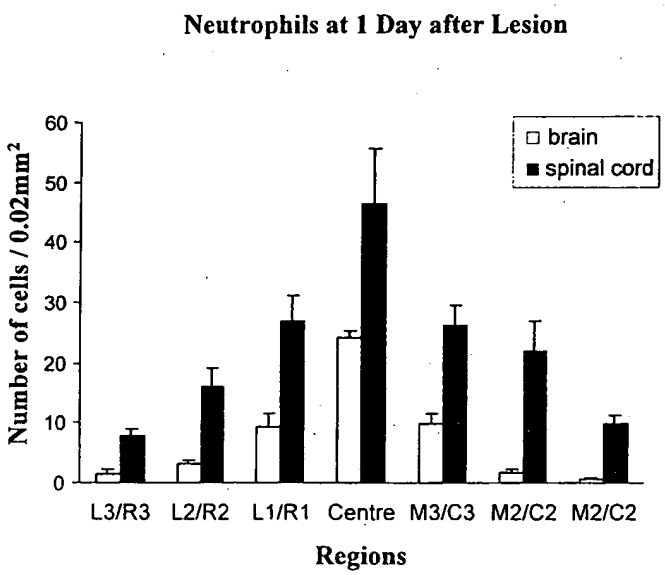

FIG. 3. Quantification of neutrophils at 1 day after lesion. Comparison of neutrophil numbers in standardized fields lateral (L, brain) and rostral (R, spinal cord) or medial (M, brain) and caudal (C, spinal cord) of the lesion, as shown in Fig. 1a and b. Values are means ± SEM of a minimum of four animals.

expressed by these cells, were used (dilutions: F4/80: 1 : 1000; FA/11: 1 : 20).

Lymphocytes

Rat monoclonal antibodies directed against mouse CD4 (T helper lymphocytes), CD8 (cytotoxic/suppressor T-lymphocytes) and B220 (CD45R on B-lymphocytes) were used. These antibodies were a kind gift of the laboratories of Dr R. Zinkernagel and Dr B. Odermatt (University of Zurich), and were diluted as follows: CD4, 1 : 6000; CD8, 1 : 10 000; B220, 1 : 50.

Astrocytes

Glial fibrillary acidic protein (GFAP) was detected with the commercially available polyclonal antibody (DAKO, UK) at a dilution of 1 : 2000.

Cell adhesion molecules

For the intercellular adhesion molecule ICAM-1 a rat monoclonal antibody (YN1/1.7.4 supernatant, American Type Culture Collection, Rockville, MA, USA) was used at a dilution of 1 : 20. For the vascular cell adhesion molecule VCAM-1 a rat monoclonal antibody (M/K2.7, ATCC) was diluted 1 : 20, and for the platelet-endothelial cell adhesion molecule PECAM, a purified rat monoclonal antibody (MEC 13.3, Cambridge Bioscience, Cambridge, UK), was employed at a dilution of 1 : 1000.

Secondary and tertiary antibodies

For neutrophils, macrophages, astrocytes, cell adhesion molecules: secondary biotinylated rabbit anti-rat (Vector Laboratories, Peterborough, UK), diluted 1 : 200, or secondary biotinylated goat anti-rabbit (Vector Laboratories), diluted 1 : 200. For lymphocytes: secondary alkaline phosphatase-conjugated goat anti-rat (Tago, Burlingame, CA, USA), diluted 1 : 75, and tertiary alkaline phosphatase-conjugated donkey anti-goat (Jackson Immunoresearch Laboratories, Westgrove, PA, USA), diluted 1 : 75.

Blocking sera

Ten per cent normal goat serum (Vector Laboratories) in phosphate-buffered saline (PBS) was used for polyclonal antibodies.

Ten per cent normal rabbit serum (Vector Laboratories) in PBS was used for monoclonal antibodies

Analysis and quantification

Cell counts for neutrophils and microglia/macrophages

Brain sections, coronal plane: using a Leitz microscope and 400 × magnification (10 × ocular with a graticule and 40 × objective), neutrophils and activated macrophages/microglia were counted. All labelled cells within seven squares of 0.02 mm^2 each were counted, with the first square (C) covering the centre of the lesion and three squares of equal size extending medially (M1–M3) and laterally (L1–L3) from it (Fig. 1a). A region approximately half way through the dorso-ventral extent of the lesion was chosen, and these areas therefore traversed the grey matter of the brain perpendicular to the

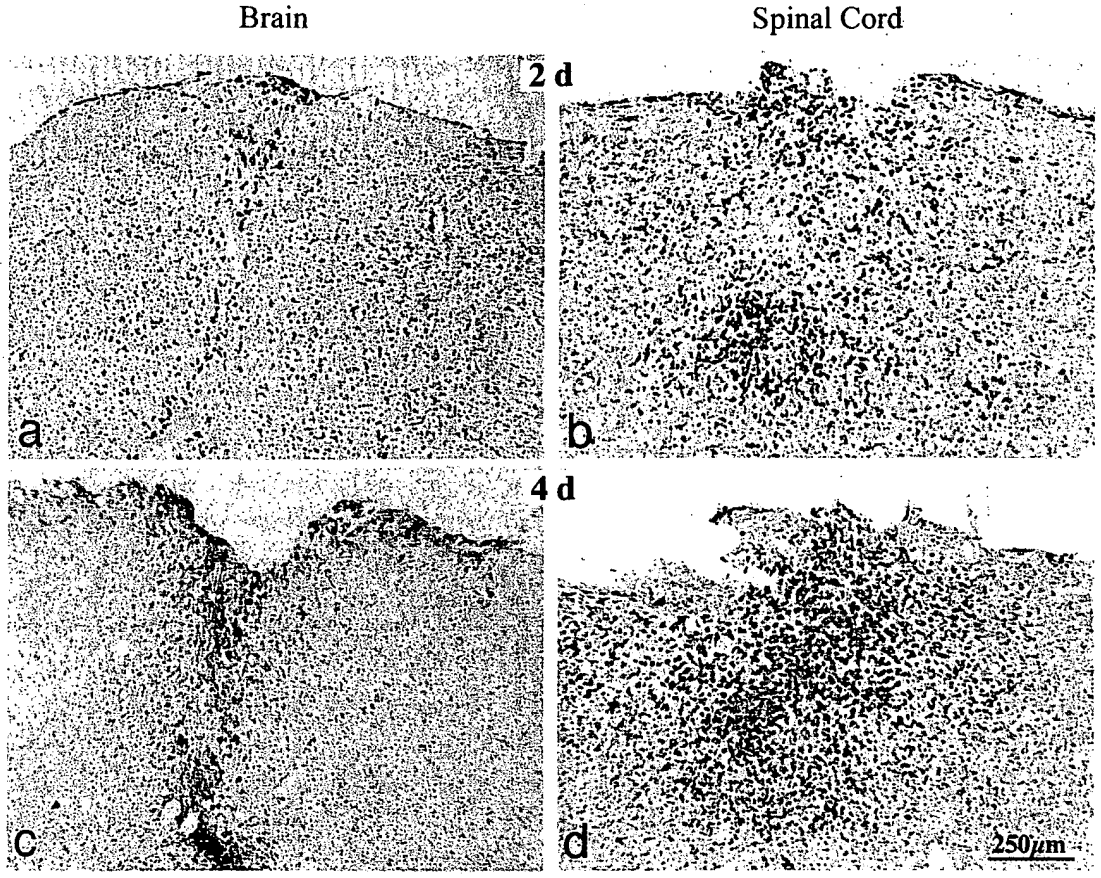

FIG. 4. Activation and recruitment of macrophages/microglia in the brain and spinal cord. Macrophage/microglia are stained with the monoclonal antibody FA/11 on 15 μm coronal sections of the brain (a and c) and parasagittal sections of spinal cord (b and d) at 2 days (a and b) and 4 days (c and d) after the lesion.

axis of the lesion. Three sections from each brain were analysed and averaged. The mean value from the combined data of at least four animals per time point was calculated.

Spinal cord sections, sagittal plane: the same counting method was used as above. The centre square (C) contained the lesion area, and the adjacent squares were located rostrally (R1–R3) and caudally (C1–C3) to it, covering the grey matter of the spinal cord (Fig. 1b).

The cell counts of brain versus spinal cord were compared using the Student's *t*-test for unpaired samples, with *P*-values <0.05 considered as significant.

Lymphocyte count

In both brain and spinal cord tissue, the number of lymphocytes was evaluated over the section containing the centre of the lesion. The very clear staining and low number of lymphocytes allowed the counting of every cell: three tissue sections of 20 μm thickness were evaluated for each animal.

Results

In this study the brains or spinal cords of adult C57BL/6J mice (total $n=88$) were subjected to focal lesions made in a standardized fashion. Spinal cord lesions crossed the dorsal aspect of the cord at the thoracic level, through the dorsal funiculi and dorsal grey matter, frequently including the central canal. Alternatively, a longitudinal cut was made in the right frontal cortex, taking care not to cut deeper than the level of the corpus callosum, although in some animals the lesion was found to extend into the underlying ventricle. In these cases the cellular response, especially the recruitment of macrophages and neutrophils, was locally enhanced in the supraventricular zone, but not in the regions used for quantification. In both spinal cord and cortex, the lesion involved both grey and white matter although, in the spinal cord, the relative proportion of white matter involved was higher.

Special care was taken in this study to avoid excessive haemorrhage (see Materials and methods). Animals with extensive bleeding (from deep, medio-ventral spinal cord vessels) were excluded from the study ($n=7$), as it was observed that extensive haemorrhage was accompanied by a higher number of macrophages and neutrophils recruited. Thus, only animals with similar lesions and minimal haemorrhage were included for quantitative analysis.

Neutrophil recruitment

Marked differences between the brain and spinal cord were observed in terms of both the numbers and distribution of neutrophils recruited by day 1 post-lesion (Figs 2 and 3). In the brain, a small cuff of neutrophils was restricted to the margins of the lesion site (Fig. 2a and c). In clear contrast, neutrophils in the spinal cord showed extensive infiltration of the neighbouring parenchyma (Fig. 2b and d). In the spinal cord, the centre of the lesion contained twice as many neutrophils as in the brain, but the difference was even more pronounced for the surrounding parenchymal infiltrate where the spinal cord contained four times the number of neutrophils (Fig. 3). The general pattern of recruitment over time, however, was similar in both locations: neutrophil recruitment peaked 1 day after lesioning and then declined rapidly thereafter. By 4 days only occasional neutrophils could be seen in the vicinity of both brain and spinal cord lesions (data not shown).

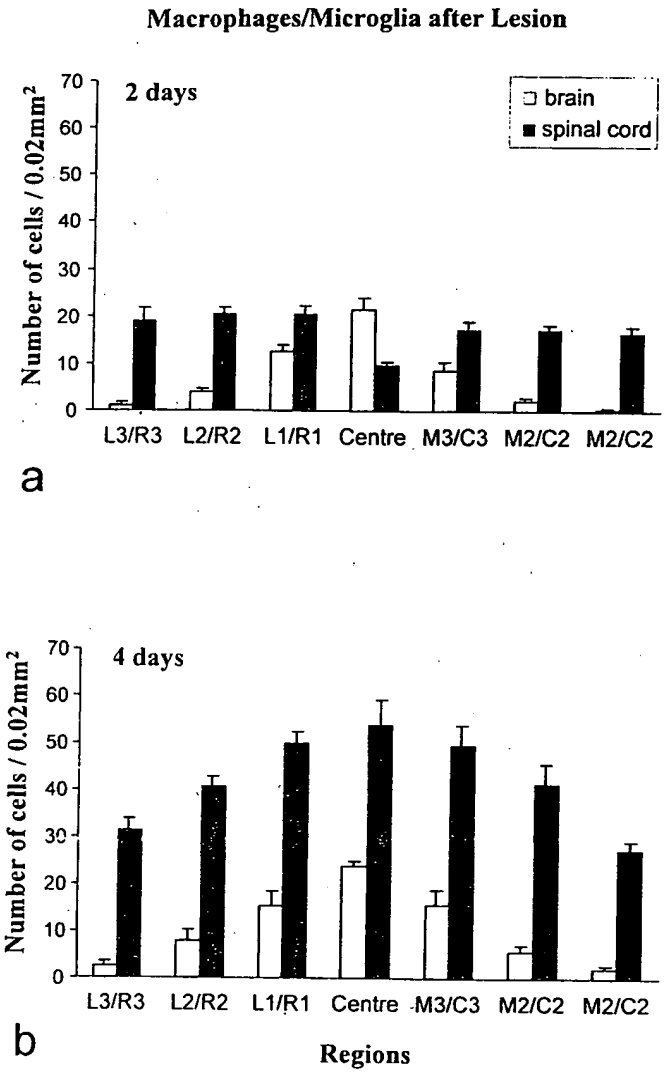

FIG. 5. Macrophage/microglia numbers in standardized areas at and around the lesion site (Fig. 1a and b) in the spinal cord and brain, 2 days (a) and 4 days (b) after the lesion. Means ± SEM of at least four animals are given.

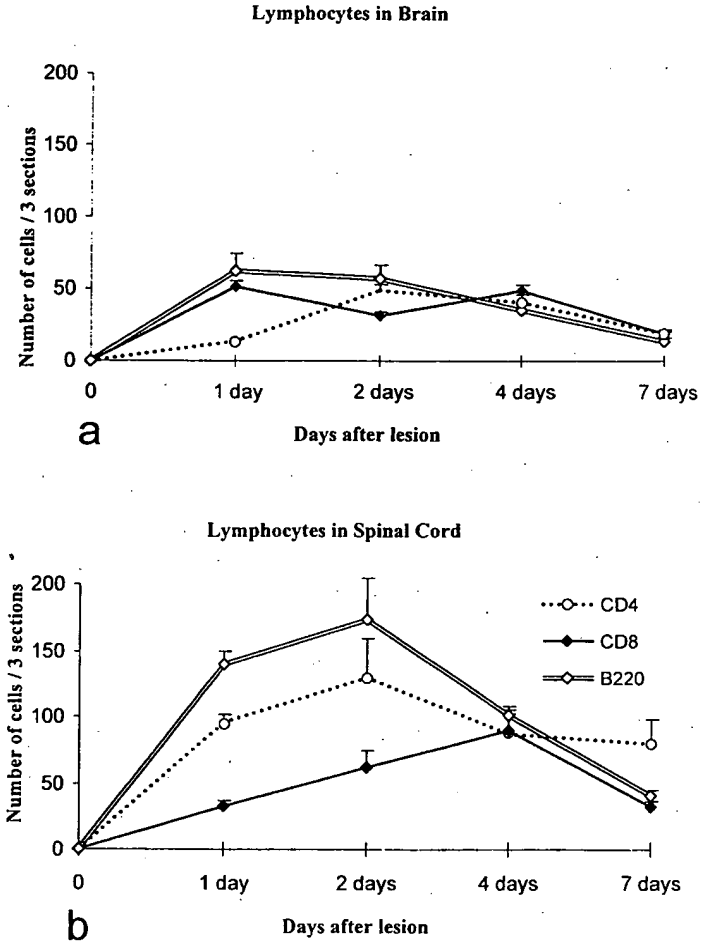

FIG. 6. Numbers of lymphocytes within CNS tissue as a function of time after lesion. The mean value of cells counted in three 20 µm coronal sections of the brain (a) or parasagittal sections of spinal cord (b) was calculated in $n=4$ (brain) and $n=5$ (s.c.) animals for each time point.

Microglia/macrophages

Both antibodies used to label activated microglia/macrophages (F4/80, FA/11) revealed a lesion-related change in the expression of these markers with similar temporal characteristics in both the brain and spinal cord. Activation of a few resident microglial cells in the lesion vicinity was evident at day 1, with the number of reactive cells gradually increasing throughout the 7 days examined. At days 2 and 4, the distribution and number of activated and recruited microglia/macrophages in grey matter were markedly different in the brain and spinal cord (Figs 4 and 5). In the cerebral cortex, the macrophage/microglia response was largely restricted to the area of the primary lesion at day 2 (Figs 4a and 5a), similar to the pattern of neutrophil recruitment, while parenchymal infiltration by these cells had increased by day 4 (Figs 4c and 5b).

In the spinal cord, the response was very widespread at day 2 (Figs 4b and 5a) and doubled again by day 4 (Figs 4d and 5b). Thus, the mononuclear phagocyte response to spinal cord injury was considerably greater than that seen following a similar-size lesion in the forebrain. It should be noted that neither FA/11 nor F4/80 distinguishes resident cell activation and proliferation from monocyte recruitment. Indeed, under the circumstances of this study, its seems plausible that there is a mixture of the two.

Lymphocytes

Previously, results in rat and mouse spinal cord showed that lymphocytes are recruited to the injured parenchyma (Popovich *et al.*, 1997; Schnell *et al.*, 1997). Here, we compare this recruitment following cord and brain lesions. CD4+ T-helper cells, CD8+ cytotoxic/suppressor T cells and B cells, were found at 1, 2, 4 and 7 days post-lesion in both of these CNS tissues, with two to three times more cell numbers in the spinal cord (Fig. 6b) than in the brain (Fig. 6a). In the brain, lymphocyte recruitment was restricted to the immediate lesion vicinity, and only very rarely could lymphocytes be detected in other brain regions. In the spinal cord, some lymphocytes could be seen as far as 3–4 mm rostral or caudal to the lesion. In comparison with other inflammatory cells recruited, the number of lymphocytes remained relatively low at all time points investigated.

Astrocytes

Twenty-four hours after lesioning, a pronounced astrocyte response was present in both spinal cord and brain tissue as defined by the upregulation of glial fibrillary acidic protein (GFAP) and thickening of cellular processes. At this time point, spinal cord tissue showed a stronger astrocyte reaction than brain tissue, a difference augmented

by day 2 (Fig. 7). In the spinal cord, astrocyte processes often acquired a sheath-like structure, especially in white matter areas (Fig. 8b), while in grey matter areas their processes kept their parallel alignment (Fig. 8). Astrocytes in grey matter areas of the brain maintained a stellate appearance (Fig. 8a). The region of astrocyte activation in the brain grey matter was largely restricted to the vicinity of the lesion, encompassing a slightly larger area than the maximal extent of neutrophil infiltration. White matter tracts, however, contained numerous activated astrocytes in both the brain (corpus callosum) and spinal cord (dorsal funiculi). In the spinal cord, activated astrocytes were observed over a much larger area than was seen in the brain, exceeding slightly the area of microglia/macrophage activation.

Cell adhesion molecules

Because we have observed marked quantitative differences in the recruitment of myelo-monocytic cells to spinal cord and brain lesions, we were interested in whether these differences might be reflected by the differential expression of vascular cell adhesion molecules. We examined ICAM, VCAM and PECAM in both the spinal cord and brain of lesioned animals and unlesioned controls.

The intercellular adhesion molecule ICAM-1 was moderately expressed in normal adult brain tissue by meningeal blood vessels as well as some very large vessels in the brain parenchyma (not shown). In the normal spinal cord, meningeal vessels and especially venous blood vessels of the posterior fissure expressed ICAM-1. One day after the lesion, ICAM-1 was already upregulated on the endothelial cells of blood vessels in the vicinity of the lesion, to a similar degree in both the brain and spinal cord (Fig. 9a and b). Subsequently, on days 2–7, expression remained elevated in both the brain and spinal cord.

In contrast to ICAM, the expression of the vascular cell adhesion molecule VCAM-1 was largely unaffected by lesioning. Up to the first 2 days after the lesion, the expression remained at constitutive levels with weak expression on meningeal arteries and some smaller vessels in the parenchyma of both brain and spinal cord tissue (Fig. 9c and d). At days 4 and 7, there was some increased expression on large and small vessels, but only in the immediate vicinity of the lesion site.

The platelet-endothelial cell adhesion molecule PECAM was constitutively expressed by endothelial cells in both the brain and spinal cord, but with particularly strong expression in the sulcal vessels of the cord. After the lesion (Fig. 9e and f), PECAM was rapidly and heavily upregulated in the spinal cord on parenchymal vessels adjacent to the lesion. In contrast, expression of PECAM in the brain remained at constitutive levels at this time point and reached spinal cord levels only at day 2. In both tissues, small vessels seemed to react stronger than large vessels, and venules more than arterioles.

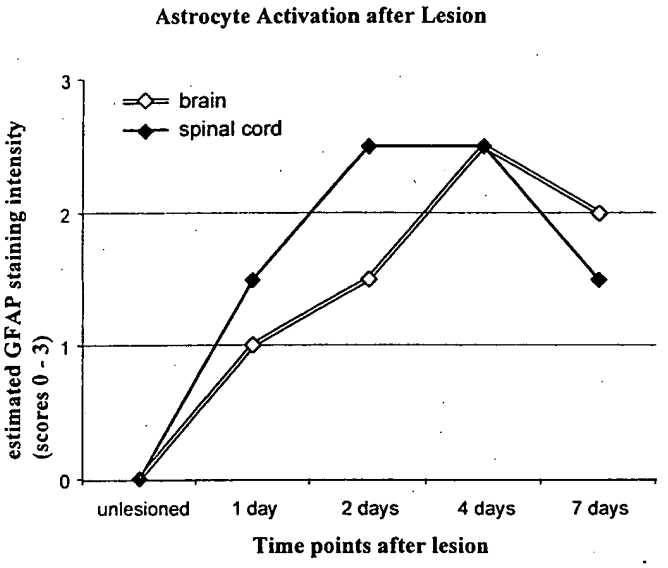

FIG. 7. Semiquantitative evaluation of astrocyte activation close to the lesion site as a function of time after brain and spinal cord lesions. Scores indicate the GFAP staining intensity from constitutive levels (0) to highest expression (3). A minimum of three animals was evaluated for each time point.

BBB permeability

HRP (5%, 100 μL) was injected intravenously 30 min prior to death. In both CNS compartments, the breakdown of the BBB was greatest at day 1 after the lesion, declining gradually thereafter. Comparison of lesion sites in the brain and spinal cord revealed a clear difference in the extent of HRP extravasation. The area of tissue affected by the BBB breakdown in the spinal cord was two to three times greater than in the cerebral cortex (Figs 10 and 11). This apparent difference in permeability was not simply due to the greater spread of HRP along white matter tracts in the spinal cord as HRP was equally present in both grey and white matter (Fig. 10). The duration of BBB breakdown also

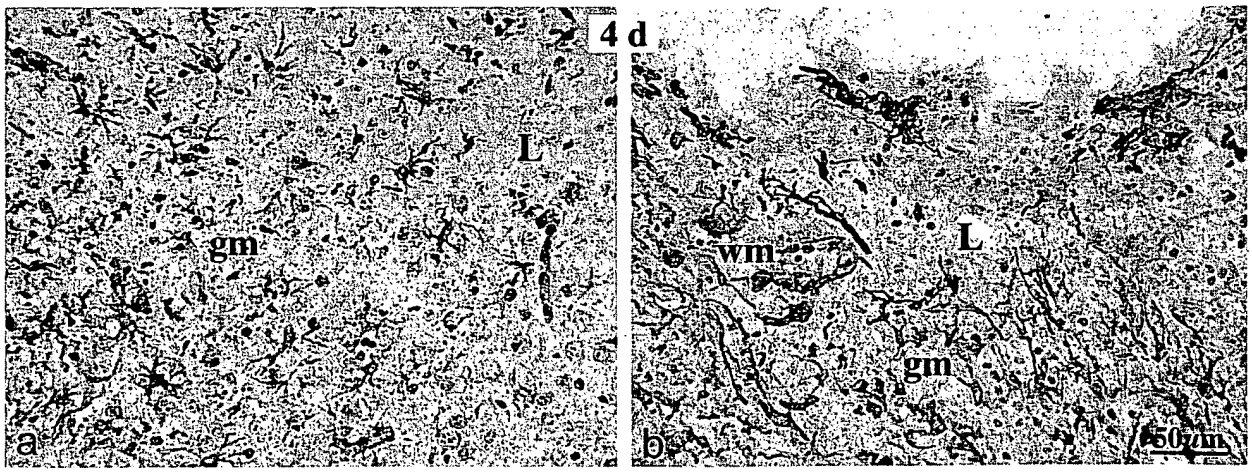

FIG. 8. Astrocyte activation in the brain (a) and spinal cord (b) as revealed by immunohistochemical staining for GFAP 4 days after lesion (L). Note the difference in morphological appearance of astrocytes in grey matter (gm) versus white matter (wm) within the spinal cord.

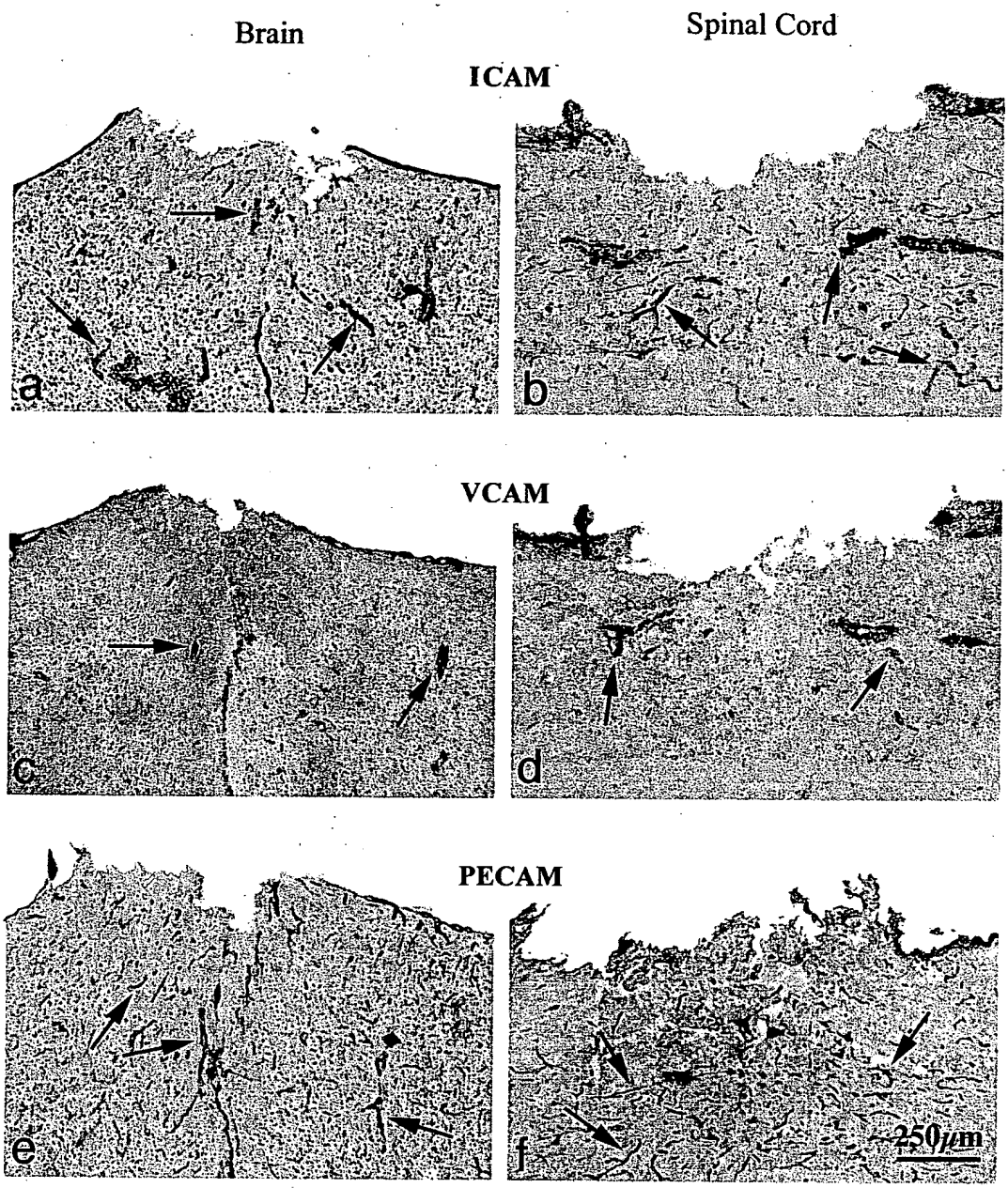

FIG. 9. Vascular cell adhesion molecules at 1 day after lesion. Fifteen-micrometre coronal sections of brain (a, c and e) and spinal cord (b, d and f), including the lesion site. Arrows point to immunoreactive blood vessels for I-CAM (a and b), V-CAM (c and d) and PECAM (e and f). Although PECAM is expressed constitutively in the CNS, enhanced expression in the lesion vicinity is clearly visible at this time point after lesion.

differed significantly: increased permeability to HRP was still observed in the spinal cord at 14 days after lesioning (latest time point examined), whereas in the brain no overt extravasation was observed after day 10 (Figs 10 and 11).

Discussion

Mechanical lesions of the CNS parenchyma result in an acute inflammatory response at the site of the lesion. Initially, this response is dominated by the recruitment of neutrophils, peaking at day 1 post-injury, and by the breakdown of the BBB. Cell adhesion molecules, e.g. ICAM-1 and PECAM are rapidly upregulated on the vascular endothelium. From day 2 onwards, blood-borne macrophages and activated resident microglia dominate the inflammatory response. Lymphocytes also appear, albeit in small numbers. Astrocytes in the vicinity of the lesion react with upregulation of GFAP and morphological changes. Interestingly, when comparing this inflammatory reaction in the forebrain and the spinal cord of adult mice, we found that the extent and severity of the inflammatory reaction in the spinal cord greatly exceeded that seen in the brain at all time points analysed.

Neutrophils in traumatic CNS lesions

In non-neuronal tissues, neutrophils are generally the first inflammatory cells to arrive at a site of trauma or bacterial invasion. Their bactericidal and phagocytic properties are crucial for the removal of both microbial intruders and tissue debris, as well as the re-establishment of homeostasis (Clark, 1993). Their appearance in the CNS parenchyma after a lesion is therefore not surprising and has been described in different models of CNS trauma (Clark *et al.*, 1994; Dusart & Schwab, 1994; Soares *et al.*, 1995). Surprisingly, however, the results of our studies revealed that the number of neutrophils

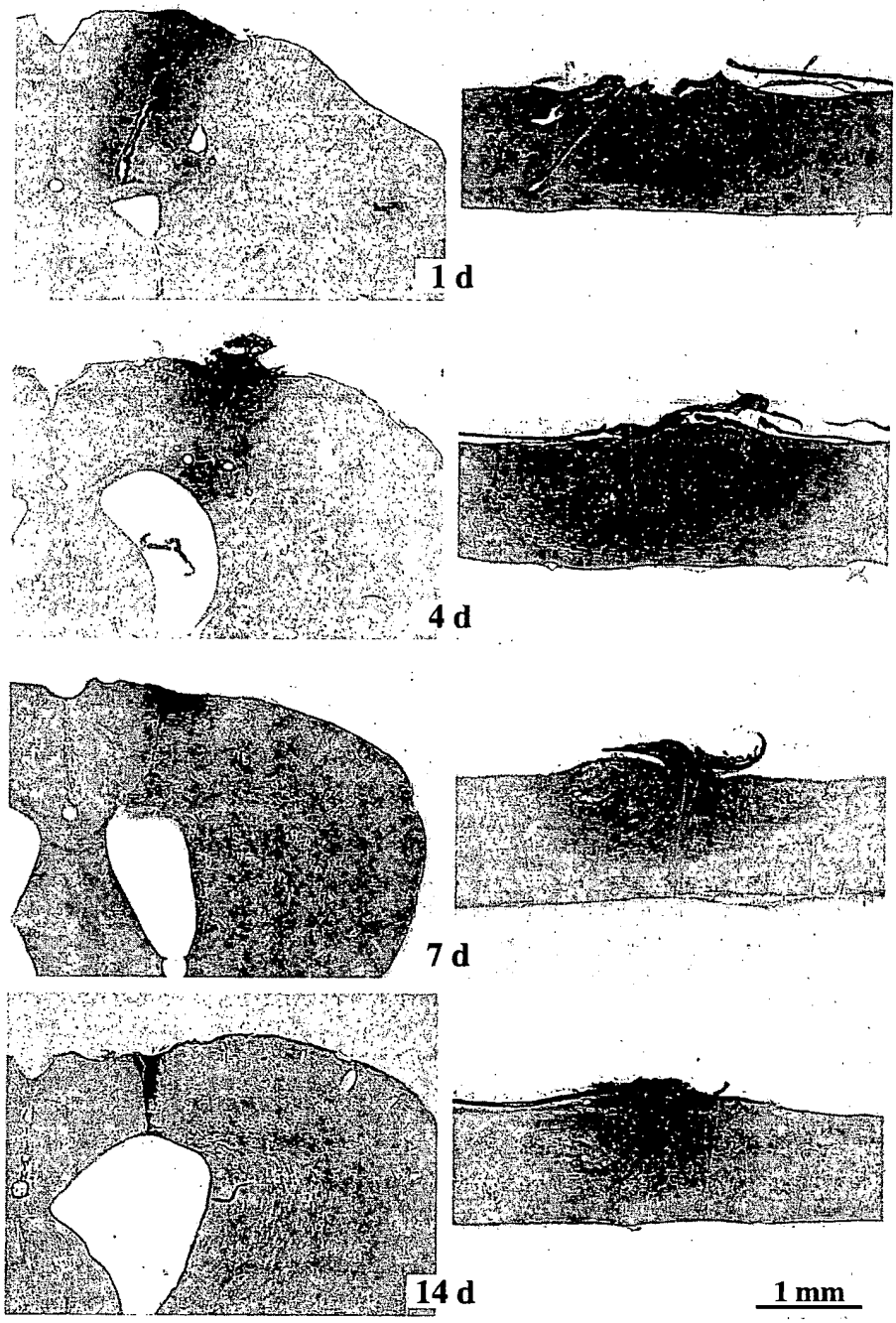

FIG. 10. BBB breakdown revealed by HRP extravasation in 30 µm coronal brain sections (left panels) and parasagittal spinal cord sections (right panels). In the brain, the re-establishment of the BBB has occurred at 14 days after the lesion. In the spinal cord the vessels are still highly permeable at this time point.

recruited to the spinal cord was significantly greater than in the brain, and the distribution of this infiltrate within the parenchyma was much more widespread.

To investigate potential mechanisms for this disparity in the cellular response pattern between CNS compartments, we examined the expression of cell adhesion molecules (CAMs) required for leukocyte adhesion to the vascular endothelium. ICAM-1 (Simmons *et al.*, 1988) and PECAM (Albelda, 1991) were indeed upregulated on blood vessels in the vicinity of the lesion in both CNS compartments. The upregulation of PECAM in the spinal cord, however, preceded that in the brain by 24 h. Furthermore, both CAMs were found to be strongly expressed by endothelial cells of the ventro-medial blood vessels of the spinal cord, even in untraumatized animals. Thus, the greater degree of neutrophil recruitment in the cord could be related, at least in part, to this difference in CAM expression.

Active tissue invasion by inflammatory cells requires not only the presence of the appropriate adhesion molecules, but also that of chemokines to attract the cells to their target. A recent study examining mRNA expression of MIP-2, a C-X-C chemokine involved in neutrophil recruitment, failed to demonstrate upregulation of this particular chemokine in response to a spinal cord lesion. In contrast, mRNA expression for macrophage inflammatory proteins MIP-1α and MIP-1β occurred as early as 45 min after trauma (Bartholdi & Schwab, 1997). Other members of the C-X-C chemokine family have not been studied in the spinal cord, but are known to be expressed in the brain following ischaemic lesions (Kim *et al.*, 1995). The possible roles of chemokines and cytokines in the differential recruitment of neutrophils to the brain and spinal cord requires further investigation (see Ransohoff & Tani, 1998 for review).

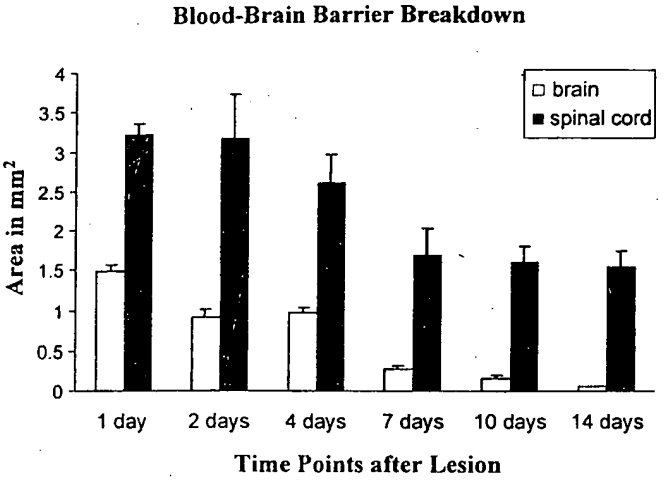

FIG. 11. Area of BBB breakdown in mm^2 as a function of time after lesion in the brain and spinal cord. Means ± SEM of three to five animals per time point.

The phagocytic activity of neutrophils plays an important role in damaged tissue, but activated neutrophils are also known to produce an array of inflammatory mediators including proteases, cytokines, chemokines and free radicals (Cassatella, 1995; Kunkel *et al.*, 1995; Conner &Grisham, 1996). The release of these mediators could result in a variety of secondary effects including the recruitment and activation of other inflammatory cells, neuronal injury, and glial cell activation (Yong, 1996). Given the results of our study, such secondary effects would presumably be more pronounced in the spinal cord than in the brain. Neutrophil depletion might therefore be a potential means of minimizing both lesion development and glial scar formation in the injured spinal cord (Schoettle *et al.*, 1990; Clark *et al.*, 1996). Indeed, the experimental application of this strategy should help to better define the pathophysiological role of neutrophils in lesioned spinal cord and brain (L. Schnell, S. Fearn, M. E. Schwab & V. H. Perry, unpublished results).

Macrophages/microglia

As for neutrophils, we found much larger numbers of macrophages in lesioned spinal cords than in brains, and a disparity in the pattern of their distribution as well. In the brain, macrophages were largely restricted to the lesion site. In the spinal cord 2 days after lesioning, activated cells were abundant up to several millimetres rostral and caudal to the lesion. Some of these cells probably represented transformed or activated resident microglia. Because it is not possible to reliably distinguish activated microglial cells from activated monocyte-derived macrophages using immunohistochemistry alone, the origin of the cells seen in our study can not be established using the methods employed. Both types of phagocytic cells, however, require the presence of appropriate signals for their activation and guidance to the lesion site. In fact, adhesion molecules necessary for the adherence of monocytes to the cerebral endothelium are expressed after CNS lesions (Hamada *et al.*, 1996). The expression of MIP-1α, a C-C chemokine which attracts macrophages, has also been shown to be transiently upregulated within the first few hours after a spinal cord injury (Bartholdi & Schwab, 1997). Prominent macrophage invasion is not, however, evident until 48 h after the lesion, a time point which correlates with the expression of MCP-1, a protein produced by macrophages and microglia in response to cerebral ischaemia (Kim *et al.*, 1995).

A key role of macrophages is the removal of tissue debris by phagocytosis. In the CNS, as opposed to the periphery, macrophages can persist in areas of Wallerian degeneration for several months (Dusart & Schwab, 1994; Schwab & Bartholdi, 1996). Macrophages can secrete an array of cytokines, e.g. IL-1, TNF and MIP, which influence a variety of other cell types. The prolonged presence of active macrophages in CNS tissue could therefore result in a number of secondary effects, either beneficial or deleterious to functional neural recovery (Schwab & Bartholdi, 1996). For instance, the activation of glia may lead to the production of growth factors essential for neuronal survival and tissue repair (Lindholm *et al.*, 1990; Maysinger *et al.*, 1996; Banner *et al.*, 1997). In contrast, the prolonged release by macrophages of pro-inflammatory cytokines and/or free radicals may contribute to subsequent exacerbation of the initial tissue damage (Mallat & Chamak, 1994; Tator *et al.*, 1995; Chao *et al.*, 1996). The extent to which macrophage activation results in either beneficial repair mechanisms or exacerbation of the lesion as a result of bystander damage remains to be elucidated.

Blood–brain barrier

Using intravascular HRP to assess vascular permeability, we determined that the region of extravasated HRP around the lesion was two to three times greater in the spinal cord than in the brain. In both CNS compartments, the BBB remained disrupted for several days after lesioning. In the brain, re-establishment of the BBB occurred ~10 days post-injury. In the spinal cord, however, the vessels remained highly permeable at 14 days, the latest time point studied.

Breakdown of the BBB after mechanical injury can to a certain extent be attributed directly to mechanical trauma of the vasculature. Acutely disrupted vessels allow the extravasation of blood cells and plasma proteins into the parenchyma. Trauma-activated endothelial and glial cells also release several species of reactive molecules, e.g. reactive oxygen radicals, bradykinins, histamines and nitric oxide (Wisniewski & Lossinsky, 1991; Azbill *et al.*, 1997). By acting directly on the endothelial cells, these molecules can enhance the disruption of the BBB (Chan *et al.*, 1984). Furthermore, massive neutrophil adhesion and diapedesis could also lead to increases in blood vessel permeability (Del Maschio *et al.*, 1996; Anthony *et al.*, 1997; Bolton *et al.*, 1998). In this regard, it is interesting to note that both the maximal number of neutrophils and the maximal extent of BBB breakdown were seen at 1 day after lesioning (the earliest time point studied). The region of increased vascular permeability corresponded closely to the sites of neutrophil recruitment. Previous studies in other models of CNS inflammation showed a similar relationship between the recruitment of neutrophils and BBB breakdown (Andersson *et al.*, 1992; Bell & Perry, 1995). Activated neutrophils also produce chemoattractants for macrophages (Ben Baruch *et al.*, 1995; Kilgore *et al.*, 1997; Ogata *et al.*, 1997), cells found in lesioned areas beginning on day 2. The diapedesis of these cells into the CNS parenchyma could also contribute to the persistence of increased vascular permeability. Thus, sustained failure of the BBB in the setting of trauma, especially in the spinal cord, may be a consequence of endothelial cell–leukocyte interactions in concert with specific chemokine expression.

An additional point to consider is the process of vascular repair and neo-vascularization. Enhanced expression of vascular-endothelial growth factor (VEGF) starting at 1 day after the lesion has been shown in the mouse spinal cord (Bartholdi *et al.*, 1997). Vessel sprouting and establishment of a new network of capillaries occurs within 1 week after spinal cord injury (Imperato-Kalmar *et al.*, 1997). These immature vessels remain leaky for some time and could

therefore represent another source of prolonged extravasation of blood proteins. The maintenance of BBB integrity by CNS vessels depends on a close interaction with astrocytes (Janzer & Raff, 1987; Hayashi *et al.*, 1997). After a lesion, astrocytes become activated but do not migrate into the lesion area (Imperato-Kalmar *et al.*, 1997). In the mouse spinal cord, however, and in contrast to the brain, the infiltration of fibroblasts into the lesion (Klusman & Schwab, 1997) could create an additional impediment to astrocyte migration and thereby delay re-establishment of the BBB.

There are important anatomical differences between the brain and spinal cord with respect to the vascular arrangement. Of note, leukocytes are preferentially recruited via the venous blood system and only occasionally from the arterial side. In the rat spinal cord, veins entering from the anterior median sulcus are almost twice as numerous as arteries (Koyanagi *et al.*, 1993). These vessels constitutively express the relevant adhesion molecules ICAM-1, VCAM-1 and PECAM. The vasculature of the rat brain follows a very different pattern with a roughly equal proportion of veins and arteries. If the same anatomical considerations also apply in the mouse, these spinal veins could represent an important source of recruited cells.

Differences in vascular anatomy could also be important for the phenomena described here in another respect: in the forebrain cortex, collateral supply is known to be rather extensive. Thus, the ischaemic zone resulting from blood vessel disruption from the primary lesion could be smaller in the cortex than in the spinal cord. Ischaemia itself may be a trigger factor for the molecular cascade leading to the recruitment of inflammatory cells. Ischaemia is probably also a crucial determinant of the secondary tissue loss observed after CNS trauma.

Conclusions

Our results demonstrate a marked difference in the reactive inflammatory events occurring within the two major CNS compartments, i.e. the brain and spinal cord. After a similar mechanical lesion, the spinal cord responds with a much more widespread activation and recruitment of inflammatory cells, as well as a longer-lasting increase in vascular permeability. The consequences of this more robust inflammatory response remain to be elucidated. The area of secondary cell death appearing shortly after the primary injury could be related to the area to which inflammatory cells were recruited. Further studies in which circulating leukocytes have been depleted, combined with quantitative evaluation of the extent of secondary damage, could provide much additional information about the potential role of inflammation in exacerbating CNS lesions.

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Abbreviations

BBB, blood–brain barrier; CAMs, cell adhesion molecules; CNS, central nervous system; GFAP, glial fibrillary acidic protein; HRP, horseradish peroxidase; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PLP, periodate-lysine paraformaldehyde; SPF, specific pathogen-free; VEGF, vascular-endothelial growth factor.

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